DOCTOR OF PHILOSOPHY

Investigation of Silver Enhanced Dental Restorative Materials by the Development of an In Vitro Oral Biofilm Model

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INVESTIGATION OF SILVER ENHANCED DENTAL RESTORATIVE MATERIALS BY THE DEVELOPMENT OF AN IN VITRO ORAL BIOFILM MODEL

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(شَهِدَ اللَّهُ أَنَّهُ لَا إِلَهَ إِلَّا هُوَ وَالْمَلَائِكَةُ وَأُولُو الْعِلْمِ قَائِمًا بِالْقِسْطِ لَا إِلَهَ إِلَّا هُوَ الْعَزِيزُ الْحَكِيمُ) ال عمران (18)

Allah (Himself) is witness that there is no God but He. And the angels and those endued with knowledge (too are witness). Maintaining His creation in justice, there is no God but He, the Almighty, the Wise. Ali 'Imran (18)

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DEDICATION

To

My Father

(Radwan Almanaseer)

My Role Model in success and courage, who gave me an amazing unconditional love and support.

My Mother

(Muna Almanaseer)

A strong and gentle soul who taught me to trust in Allah, believe in hard work and that so much could be done with little.

My Husband and Children

(Mohammad Albtoon) (Malak, Ali and Nash)

The secret of happiness and the sunshine of my life.

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A special thanks to my beloved family. Words cannot express how grateful I am to my father and to my mother for all of the sacrifices that you've made on my behalf. Your prayer for me was what sustained me thus far. My brothers and sisters for their love and support. I would like to thank my husband for the happy moments we had together. Also my father and mother in law for being in my life. I would also like to thank all of my friends who supported me through this journey, and incented me to strive towards my goal. I would like express my sincere love to my children; Malak, Ali and Nash who shared with me all the good and bad moments.

I would especially like to thank all of the staff from Dundee Dental School for their endless support and collaboration. Finally, I would like to thank my sponsors; University of Jordan for giving me the opportunity to finish my studies.

DECLARATION

I declare that I am the author of this thesis and that I have consulted all the references cited. The work of which this thesis is a record has been done by myself and has not previously been accepted for a higher degree. This work has been carried out in the Microbiology, Dental Materials and Biochemistry laboratories of Dundee Dental School and Hospital, under the supervision of Professor Mark P Hector and Dr David H Edwards.

Signature …….

Date …….

Wijdan Radwan Elmanaseer

ABSTRACT

Generally, *in vitro* oral biofilm models are commonly used to help in understanding the complex processes and the factors affecting oral diseases. They help to accurately predict, in a controlled and simplified way, a clinical outcome which can lead us to preventive actions for a disease (Salli and Ouwehand, 2015).

The complexity of biofilm research requires different approaches to address various questions. Furthermore, models cannot capture all of the details involved with disease formation, however it is considered a way of performing a reproducible experiment under controlled conditions. Obviously there are ethical limitations with *in vivo* studies in relation to caries and periodontal diseases. Therefore, different *in vitro* techniques have been developed and are continuously improved to better address the study question, to help interpret the results and to obtain as much information as possible with other than clinical testing (Salli and Ouwehand, 2015).

This project aimed at developing a robust *in vitro* oral biofilm model to determine the effectiveness of enhanced dental restorative materials using antimicrobial additives. It is focused on the antibacterial and mechanical properties of novel silver formulations that have been combined with glass ionomer cement. Three out of nine bacterial species considered to be early colonisers (*Streptococcus oralis, Streptococcus mutans,* & *Neisseria subflava*) were inoculated at 0.1 OD600 into wells containing sterile glass-ionomer disks. The established artificial saliva media DMM (Defined Medium Mucin) was used for 24-48Hrs studies, conducted under aerobic and anaerobic conditions.

Following incubation, the disks were washed with PBS (Phosphate Buffer Saline) in a series of up to five, twelve minute steps. The bacterial community within the well, loosely attached to the well surface and the disk, and finally the bacteria intimately attached to the surface were determined using a viable count of Colony Forming Unit

Count (CFUs), a MTT metabolic assay and DNA quantification. Three silver solutions were developed with different ionic concentrations, 5mg/ml, 10mg/ml and 13mg/ml. The addition of polyvinyl alcohol to stabilise the 10mg/ml solution was investigated and along with the MIC/MBC and the effect on the ability of three bacterial species to survive and colonise glass ionomer disks was determined. In addition, compressive strength, hardness and adhesive shear bond strength of each glass-ionomer silver disk was assayed and compared with the base line glass-ionomer.

The biofilm model favoured survival of *S. oralis* under aerobic conditions and *N. subflava* and *S. oralis* under anaerobic conditions. Silver formulations proved to have effects on both strength and antimicrobial activity. Specifically, 5mg/ml proved more antibacterial than 10mg/ml silver. Compressive strength was enhanced for both concentrations of additive, however bond strength became compromised at the higher concentration.

This model proved its efficiency to test the antibacterial activity of silver enhanced glass ionomer cement in the presence of artificial saliva. Furthermore, addition of 5mg/ml silver to the glass ionomer cement enhanced antibacterial activity and physical properties significantly.

CERTIFICATE

I hereby certify that Wijdan Radwan Elmanaseer has fulfilled the condition of Ordinance 39 of the University of Dundee and is qualified to submit this thesis for the degree of Doctor of Philosophy in Dentistry.

Professor Mark P Hector

Dean and Boyd Professor of Dental Surgery

Dundee Dental School and Hospital

Singed

Date

Dr David H Edwards

Senior Lecturer Oral Microbiology

Dundee Dental School and Hospital

Signed

Date

LIST OF ABBREVIATIONS

µj	micro-joules
3D	Three Dimensional
Ag^+	Silver Ions.
AgNPs	Silver Nano-particles
AMM	Artificial mouth models.
ATCC	American Type Culture Collection.
ATP	Adenosine Tri-Phosphate
BHI	Brain Heart Infusion
BMM	Basal Medium Mucin
bp	base pairs
CB	Columbia Broth
CFUs	Colony Forming Units.
CHX	Chlorhexidine.
CLSM	Confocal Laser Scanning Microscopy.
CP-OCT	Cross- Polarisation Optical Coherence Tomography
DMM	Defined Medium Mucin.
DMSO	Ddimethylsulfoxide.
dNTPs	Deoxynucleoside triphosphates

EPS	Extracellular Polymeric Substances
FISH	Fluorescent In Situ Hybridisation
g	gram
GIC	Glass Ionomer Cement.
gtf	Glucosyltransferase enzyme gene.
HA	Hydroxyapatite
HOMIM	Human Oral Microbial Identification Microarray
Hrs	Hours
i.e.	That is
Ig	Immunoglobulin
Kbp	kilo base pairs
Kg	Kilogram
KHz	Kilohertz
L	Litre
Log	Logarithmic
M	Molar
MBC	Minimal Bactericidal Concentration.
Mean±SEM	Mean ± Standard Error of Mean
MIC	Minimal Inhibitory Concentration.

ml	millilitre
MSA	Mannitol salt agar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	Newton
n	number of samples
NaF	Sodium Fluoride
NCTC	National Collection of Type Cultures
OD_{600}	Optical Density at 600nm wavelength
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PMM	Part Per Million
PMMA	polymethylmethacrylate
PS	Polystyrene.
PTFE	Polytetrafluoroethylene
PVA	Polyvinyl Alcohol
PVP	Polyvinylpyrrolidone.
QAC	Quaternary Ammonium Compound.
qPCR	quantitative Polymerase Chain Reaction.
RMGIC	Resin Modified Glass Ionomer Cements.

rplF	50S ribosomal protein L6 gene.
RPM	Round Per Minute
SD	Standard Deviation
SEF	Surface Free-Energy.
SEM	Scanning Electronic Microscopy.
SR	Surface Roughness.
TAE	Tris/Acetate/EDTA Buffer.
TBE	Tris/Borate/EDTA Buffer.
TF broth	*Tanerella forsythia* broth
TM (QLF-D)	Quantitative Light-induced Fluorescence-Digital illuminator
TSB	Tryptic Soy Broth.
UV	ultraviolet
VLC	Visible light cured
X	Times

Chapter 1 INTRODUCTION

Plaque-related diseases are probably considered the most common bacterial diseases occurring in man (Allaker and Douglas, 2009). Dental caries (dental decay), which affects over 80% of the population in many countries, is a destructive condition of the dental hard tissues (Petersen and Lennon, 2004), if untreated lesions can progress to inflammation and death of vital pulp tissue, with the possibility of damage eventually spreading to the periapical area of the tooth and beyond. Caries is caused by acids (principally lactic acid) in the oral cavity which initiate demineralisation of tooth enamel, resulting in lesions that can develop into cavities. The most significant source of lactic acid is via the anaerobic metabolism of dietary sugars by bacteria associated with the oral biofilm known as 'dental plaque'. Dental caries is classified into Primary and Secondary caries depending on whether it is diagnosed on an intact enamel surface or in association with an existing restoration that has been in place for some time. Secondary caries or recurrent caries is either caused because of defective restorations (caries around restorations) or lack of preventive measures in high caries risk patients, however secondary caries is considered one of the most important aetiological factors in restoration failure and the most common reason for replacing adhesive fillings, particularly in high caries risk patients (Demarco et al., 2012, Opdam et al., 2012, Roumanas, 2010, van de Sande et al., 2013).

Dental plaque is also considered to be the primary aetiological cause of periodontal diseases which are considered the most common destructive inflammatory conditions that affects man (Allaker and Douglas, 2009). Periodontal diseases can involve both the soft and hard tissues. They are initiated by components of the plaque that develops on the hard tooth surface adjacent to the soft tissues of the supporting

periodontium and may be confined to the gingiva (gingivitis) or extend to the deeper supporting structures with destruction of the periodontal ligament and the alveolar bone that supports the teeth (periodontitis). In the most severe cases the loss of attachment, with associated periodontal pocket formation, may ultimately lead to loosening and loss of the affected teeth.

Even though the missing tooth can be replaced by a dental implant**,** the threat of bacterial communities is still capable of compromising the success and longevity of the clinical interventions. The use of dental implants has become a routine procedure in dentistry to replace one or more missing teeth. The incidence of chronic inflammation is between 8.6%-9.7%, in soft and hard tissues neighbouring implants (Guggenheim et al., 2001, Pjetursson et al., 2004), and is commonly observed about ten years after implantation (Roos-Jansaker et al., 2006). Mucositis and Peri-implantitis are considered the main complications in dental implantology with their clinical manifestations such as gingival bleeding, swelling and bone loss they strongly resemble periodontal inflammation (Guggenheim et al., 2001). The composition and mechanisms of Dental plaque formed on implant surfaces is similar to that on teeth (Quirynen et al., 1994).

However, changing the perspective in treating plaque induced oral diseases is a priority; i.e. to develop anti-microbial technologies that enhance the longevity or success of dental therapies such as dental restorations and implants.

Glass ionomer cement (GIC) is found to be the most cariostatic dental material and claimed to have an antibacterial property due to release of fluoride. However, annual clinical surveys found that secondary caries was still the main reason for GIC failure, indicating that the fluoride-release from GICs is not potent enough to inhibit bacterial growth or combat bacterial destruction (Bernardo et al., 2007).

Consequently, the traditional mechanical and non-specific methods for dental plaque control are not sufficient enough to control oral diseases and the new trend is going now towards enhancing the anti-bacterial activity of dental restorations and implants aiming to augment the existing well-known plaque control methods.

Generally, the antibacterial activity of dental materials is tested either using simple methods or by more complex oral biofilm models. Simple methods including Agar diffusion tests, Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) were the main reported testing methods in the literature (Reller et al., 2009). However, oral biofilm models are now commonly used to help in understanding the complex processes and the factors affecting the oral diseases. In addition, they helped in accurately predicting, in a controlled and simplified way, a clinical outcome which can lead to preventive actions for a disease (Featherstone, 1996).

This project is concerned in developing a robust *in vitro* oral biofilm model to determine the effectiveness of enhancing dental restorative materials with antimicrobial additives. It is focused on the antibacterial and mechanical properties of novel silver formulations that have been combined with glass ionomer cement.

Chapter 2 LITERATURE REVIEW

2.1 Oral Biofilms (Dental Plaque)

Naturally, micro-organisms tend mostly to be organised into communities, and these are found assembled on non-living or living substrates as biofilms.

The oral biofilm is one of the most complex and diverse bacterial communities associated with the human body. So far, more than 1000 different bacterial species have been identified in the human oral cavity (Aas et al., 2005, Dewhirst et al., 2010, Keijser et al., 2008, Paster et al., 2001, Paster et al., 2006), and the Human Oral Microbiome Database lists 1,200 predominant oral species, with some 19,000 phylotypes (Keijser et al., 2008).

Distinct subsets of bacterial communities predominate in different habitats (Xu et al., 2015). The majority of them are associated with dental plaque, forming highly organised microbial communities (Kuramitsu et al., 2007, Marsh, 2005).

2.1.1 Development of Oral Biofilms

2.1.1.1 Acquired Pellicle Formation

The formation of the acquired pellicle is a highly selective and dynamic process influenced by several factors, including circadian cycles, oral microbial flora composition, the proteolytic capacity of the oral environment, and the physical and chemical properties of tooth surfaces, as well as location in the mouth (Hannig and Joiner, 2006).

The initial stage of acquired pellicle formation starts within seconds of exposure to whole saliva and is characterised by an increase in pellicle thickness to 10 to 20 nm within a few minutes that remains stable for about 30 minutes (Hannig and Joiner, 2006). Salivary proteins with a high affinity for hydroxyapatite, commonly referred to as ‘pellicle precursors’, initiate this process via electrostatic interactions with the enamel surface (Hannig and Joiner, 2006, Hay, 1973). *In vitro* studies identified acidic proline-rich proteins (aPRPs), statherin, and histatins as being among the first to adsorb to

hydroxyapatite (Siqueira et al., 2012a). Besides those proteins, in situ studies have confirmed the presence of mucins (MUC5B and MUC7), amylase, cystatins, lysozyme, and lactoferrin as the most abundant proteins in the acquired enamel pellicle (Siqueira et al., 2012a).

After this first increment, a rapid increase in acquired pellicle thickness is attributed to the adsorption of protein aggregates from saliva to it by means of protein-protein interactions. Thereafter, the pellicle thickness reaches a plateau, between 30 to 90 minutes, at a thickness of 100 to 1000 nm, depending on its location within the oral cavity (Hannig and Joiner, 2006). This characterises the second or the maturational stage of pellicle formation.

2.1.1.2 Early Colonisation

Specific bacterial species initiate biofilm formation by attaching directly with the acquired pellicle (Figure 2.1). *Streptococci* spp., *Actinomyces* spp., *Capnocytophaga* spp., *Eikenella* spp., *Haemophilus* spp., *Veillonella* spp., and *Neisseria* spp., are the main pioneer colonisers which attach directly to the pellicle (Dige et al., 2009, Foster and Kolenbrander, 2004, Ritz, 1967). However, the attachment at this point is reversible and those initially attached bacteria can easily detach from the pellicle. The early attachments are primarily with electrostatic attractions or physical attachments, but later, chemical forces predominate (Rittmann and Laspidou, 2003).

Once the pioneer bacteria attach to the pellicle, they begin to excrete extra-cellular polymeric substances (EPS), which help the bacteria remain bound to each other and to the components of the pellicle. The forces of adhesion between the pioneer bacterial and salivary acquired pellicle include hydrogen bonds, hydrophobic interactions, calcium bridges, van der Waals forces, acid-base interactions and electrostatic interactions

(Hannig and Hannig, 2009). These early colonisers modify the local environment and make conditions suitable for colonisation by more fastidious organisms.

Early colonisers such as Streptococci (Kolenbrander et al., 2006) can alter the pathogenic potential of the oral biofilm through both their influence on biofilm community development, and by elevating the pathogenic potential of other bacteria (Whitmore and Lamont, 2011).

Consequently, a complex biofilm will result not only through multiple levels of contact and adhesion between bacteria, environmental components and the enamel or cementum substrate, but also via metabolic and physical factors such as access to oxygen and carbon sources.

2.1.1.3 Secondary Colonisation and Late Colonisation (Aggregation)

Secondary colonisers attach to receptors and binding sites provided by pioneer bacteria (Figure 2.1). The secondary coloniser species include *Fusobacterium nucleatum, Treponema* spp., *Tannerella forsythensis, P. gingivalis, Aggregatibacter actinomycetemcomitans,* (Foster and Kolenbrander, 2004, Dige et al., 2009, Ritz, 1967, Kreth et al., 2005, Kolenbrander et al., 2002). *Fusobacterium nucleatum* is thought to play a main role in the maturation with almost all bacteria including early colonisers and late colonisers (Figure 2.1). So, *F. nucleatum* will act as a co-aggregation bridge between the bacteria which do not naturally co-aggregate with each other.

The mechanism of co-aggregation is generally dependent on cell-to-cell recognition which allows one bacterial species to co-aggregate with many other bacterial species. Paired aggregation tests among 300 oral bacteria species indicated more than 90% undergo co-aggregation (Kolenbrander, 1995). However, aggregation is also important because some bacterial strains have very specific requirements & do not aggregate

randomly with bacterial strains, i.e., *S. mutans* aggregates with *F. nucleatum* but not with *P. gingivalis*.

Polysaccharide recognition is considered the main mechanism of aggregation between oral bacteria. The polysaccharide recognition sites vary from one paired bacterial recognition to another paired bacterial recognition, because each bacterial cell has several different receptors which are complementary to different adhesions belonging to other bacterial species.

Co-aggregation bridges usually refer to a structure of one bacterial species with two or more different receptors which can be recognised by different adhesions of two or more different bacterial species. Those bacterial species could aggregate by attaching to the first bacterial species; i.e. *F. nucleatum* is one of the best known co-aggregation bridge species that facilitates Streptococcal and obligate anaerobes aggregation (Mahajan et al., 2013, Ritz, 1967). However, on the other hand, two bacterial cells may compete for the same binding site on another bacterial cell.

2.1.1.4 Maturation

The maturation process involves proportional shift in the microbial components. The relative amount of *Streptococci* and *Neisseria* decreases, while the amount of *Actinomyces, Corynebacterium, Fusobacterium,* and *Veillonella* increases (Kolenbrander et al., 2010).

The integrity of the biofilm community is maintained by intermicrobial adhesion, cell signalling by means of cell-to-cell contact, metabolic communication, and quorum sensing (Kolenbrander et al., 2002).

The extracellular polymers continue to be synthesised by the bacterial community contributing to the biofilm matrix, which is considered to be biologically active because

it retains and binds molecules, including active enzymes and that adds an extra value to the biofilm along with it being a structural scaffold (Guo et al., 2014).

Also the metabolism of the community continues by breaking down complex host macromolecules (e.g. mucins) using combined bacterial metabolic forces in order to obtain nutrients (Guo et al., 2014). Food chains develop, and the metabolic product of one organism becomes a primary nutrient for another. To that end, these oral biofilms become functionally and structurally organised, and with a higher biological property than the sum of the individual species especially with the ability to resist antimicrobials.

2.1.1.5 Microbial Homeostasis

Microbial homeostasis is the stage of natural balance when composition of microbial communities at a site remains relatively constant over time after the biofilm is established and mature. In another words the resident oral bacterial community coexists in a harmonious relationship with the host, which contributes to the normal development and general health of the host.

This homeostasis excludes exogenous (and often pathogenic) micro-organisms, so supports the innate and adaptive host defences, and is responsible for the natural development of the physiology of the host (Kolenbrander et al., 2010). However, it is continually subjected to environmental challenges in the oral cavity including variations in oxygen and nutrient availability, pH fluctuations and the antimicrobial properties of saliva (Abiko et al., 2007).

On some occasions, this harmonious relationship can be shifted, and disease can occur. For example, in caries, there are increases in acidogenic (acid producing) and aciduric (acid-tolerating) species such as *lactobacilli* and *S. mutans* respectively (Marsh, 2009). Similarly, with periodontal disease, the subgingival biofilm has elevated levels of

proteolytic bacteria that can subvert the host inflammatory response. The predominant bacteria at inflamed sites include obligate anaerobic Gram negative bacteria, including, for example, representatives of the genera *Prevotella*, *Porphyromonas*, *Fusobacterium*, *Treponema*, and *Tannerella* (Socransky et al., 1998).

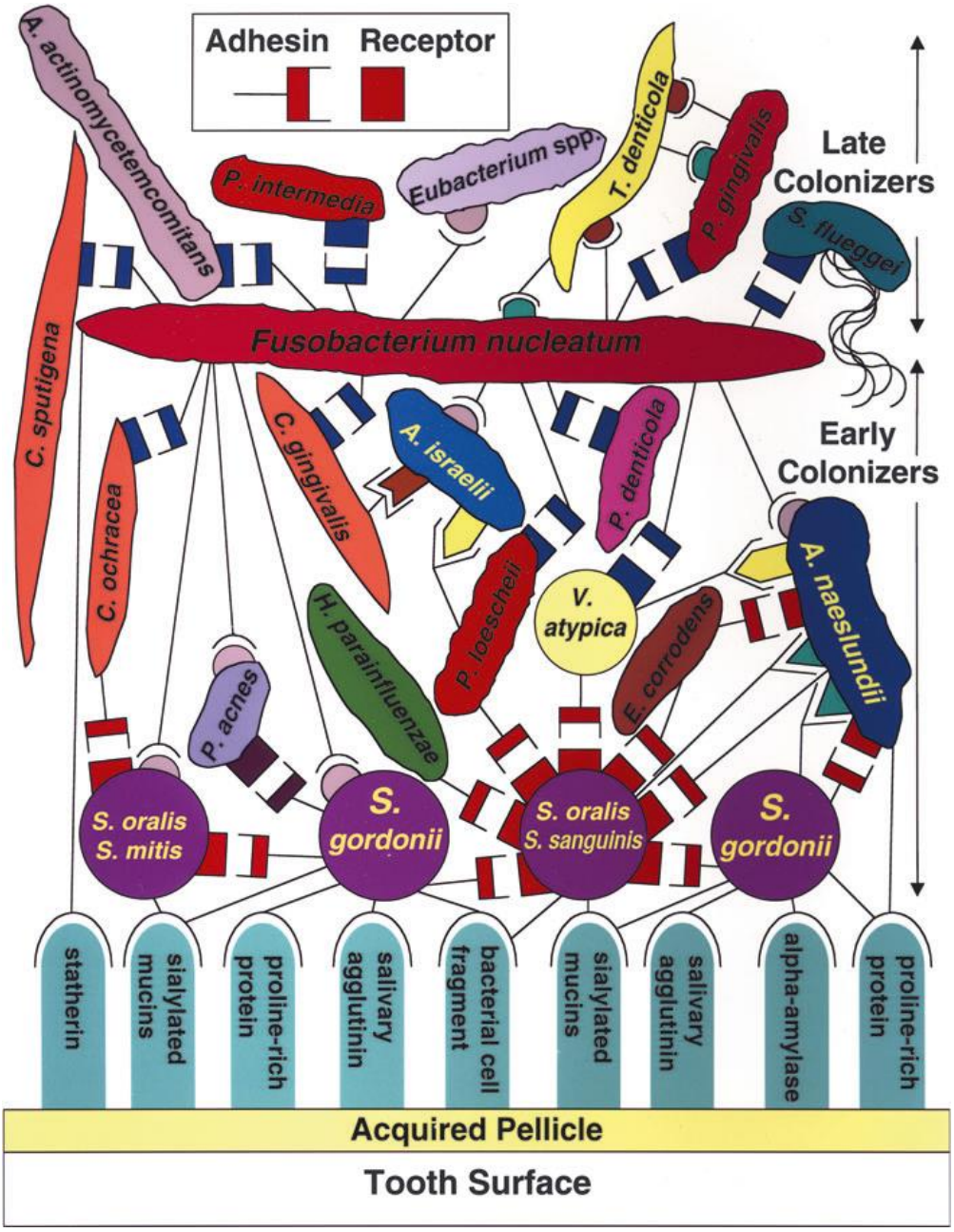


Figure 2.1 Illustrating structure of Dental plaque (Oral biofilm) formed on the tooth surface. It shows how the early colonisers attached to the acquired pellicle and then the late colonisers co-aggregate with early colonisers. It also shows the bridging role of *F. nucleatum* between early and late colonisers. Adapted from (Kolenbrander et al., 2006).

2.1.2 Aetiology of Oral Diseases

The microbial aetiology of oral and dental diseases has been extensively studied; however, the complex nature and diverse composition of oral biofilms makes it difficult to identify the causative micro-organisms.

A traditional view of plaque induced oral diseases, which was based upon clear cause-and-effect relationships, has developed into a more generalised view which allowed theories to consider that the predominance of certain microorganisms at a given site may be the result of the disease itself rather than that of the initiating agent (Socransky et al., 1998).

The ecological plaque hypothesis (Hardie et al., 1977, Marsh, 1991, Marsh, 1994, Marsh and Bradshaw, 1997, Marsh et al., 1989, Newman, 1990, Kleinberg, 2002) has improved on these classic infectious concepts to explain the aetiology of caries and periodontal disease. This hypothesis suggests that the organisms associated with the disease may also be present at healthy sites, but at low levels or in a state that is not overtly pathogenic, and therefore do not trigger a clinical pathology and the disease occurs when the there is a shift in the balance of the oral biofilm.

Despite the complexity of the oral microbial community, and the models that explain the shift from health to disease in broad terms, there remains interest in keystone pathogens with the capacity to rapidly shift and alter the behaviour of a community. For dental caries *S. mutans* and *Lactobacillus* are still concerned, for periodontal disease *Porphyromonas gingivalis*, *Tanerella forsythia*, *F. nucleatum* and other species. The role of these species in oral diseases will be explained in the next sections.

2.1.2.1 Dental Caries

Although *S. mutans* has been widely implicated and studied in the context of Caries, carious lesions can exist in the absence of *S. mutans* and be absent in its presence (Marsh et al., 1989). The accepted view for caries is that any acid-producing species capable of metabolising sugars at low pH, can contribute to the caries process. This description includes *S. mutans*, *Lactobacilli*, *Bifidobacteria*, *Rothia* and others. *S. mutans* produced extracellular polymeric substances (EPS) which modulate the three-dimensional architecture and population shifts during morphogenesis of biofilms (Xiao et al., 2012). For that reason, *S. mutans* and *S. oralis* and *L. casei* were chosen in this study as part of the *in vitro* biofilm consortium.

2.1.2.2 Periodontal Diseases

Microbes associated with periodontal disease, including *P. gingivalis*, *P. intermedia* and *Aggregatibacter actinomycetemcomitans* (Mahajan et al., 2013). However, *S. oralis* proved to play a major role in periodontitis pathogenesis because it provides a unique receptor sites for later, more pathogenic colonisers such as *F. nucleatum* (He and Shi, 2009), *T. forsythia*, *Treponema denticola* and *P. gingivalis* (Whitmore and Lamont, 2011). Therefore, *A. actinomycetemcomitans, S. oralis*, *F. nucleatum*, *T. forsythia* were chosen to be part of this project *in vitro* biofilm consortium.

2.1.2.3 Peri-implantitis

Bacteria have been identified to colonise implant surfaces in partially as well as in fully edentulous patients in a similar way to teeth.

T. forsythia, P. gingivalis, T. denticola, Prevotella nigrescens, P. intermedia, F. nucleatum, Campylobacter spp., *Parvimonas micra* and *A. actinomycetemcomitans* have been detected in peri-implantitis sites both in fully and partially edentulous patients (Busscher et al., 2010).

The colonisation of “pristine” peri-implant pockets with periodontal disease associated bacteria has been shown to occur within 2 weeks (Quirynen et al., 1994) and that some of these bacteria were found to be present as early as 30 min after insertion of the implant (Furst et al., 2007). Because biofilm formation on implant surfaces is similar in composition and mechanisms known from periodontitis (Quirynen et al., 1994). Same as periodontal causative micro-organisms; *A. actinomycetemcomitans*, *S. oralis*, *F. nucleatum*, *T. forsythia* were chosen to be part of this project *in vitro* biofilm consortium.

2.1.3 Features of Oral Biofilms on Dental Hard Tissue and Conventional Dental Restorative Materials

2.1.3.1 Dental Hard Tissues

All biofilms on the tooth surface have at least one characteristic in common; the presence of a bacterial-derived EPS that envelopes the bacteria and helps to facilitate the formation of multicellular structures that become firmly attached to the dental hard tissue (Flemming and Wingender, 2010).

The rate of colonisation is different between enamel and root surface, in the first eight hours after cleaning, enamel was sparsely colonised by multispecies communities (Kolenbrander et al., 2010), while under equal conditions root surfaces were more heavily colonised with the same types of bacteria (Nyvad and Fejerskov, 1987a, Nyvad and Fejerskov, 1987b). The mechanism of oral biofilm formation on enamel was discussed previously, i.e. early colonisation, late colonisation etc. *Streptococcus sanguinis* contributed 6–18% of the early colonisers. *Streptococcus mitis* and *S. oralis* varied between 24–42% and 1–27% respectively. The relative proportion of *S. oralis* increased significantly within 24 h while the proportion of *Streptococcus salivarius* and *S. mitis* showed a declining tendency (Nyvad and Kilian, 1987).

2.1.3.2 Ceramics

Restorative materials surfaces are different chemically and physically from tooth tissues therefore, biofilms formed over are assumed to be different. Earlier there was little attention in modelling restorative materials for microbial behaviour till the understanding of the complexity nature of oral biofilm were studied on restorative materials.

Ceramic materials are low-adhesive materials due to their inherent surface characteristics. Biofilms on ceramics are thin and highly variable (Busscher et al., 2010). Compositional and microstructural differences between different ceramics may, however, influence the

surface properties and hence the reactions between the material and oral microbial environment.

Zirconia ceramic in particular exhibits low plaque accumulation, and displays similar bacterial binding properties to titanium (Lima et al., 2008). Also ceramic collected less plaque with reduced viability under conditions of no oral hygiene than did the natural tooth surface (Hahn et al., 1993). A 6 µm thick biofilm on ceramics took five days to form *in vivo* compared to gold and amalgam which attracted up to 17 µm thick biofilms (Auschill et al., 2002).

2.1.3.3 Resin Composites and Glass-ionomer

Biofilm formation on resin composites and glass-ionomer cements led to unexpected outcomes (Beyth et al., 2008) in a way that colonising organisms cause severe deterioration of the surface, which, in turn, promotes biofilm formation and therewith more extensive deterioration of the surface, which eventually lead to development of caries around or below a restoration (Sousa et al., 2009). Surface deterioration of resin composites and glass-ionomer cements has been demonstrated by increased roughness, effects on filler particle exposure, and sometimes by a decreased micro- hardness of the materials upon exposure to biofilms *in vitro*.

2.1.3.4 Composite

2.1.3.4.1 Polishing and Biofilm Formation

Many experimental data demonstrated that high surface roughness (SR) and, to a lesser extent, high surface free-energy (SFE) of a dental-restorative material are related to increased biofilm formation on its surface (Bollen et al., 1997, Ionescu et al., 2012, Teughels et al., 2006, van Dijk et al., 1987). Thus, attempts to minimise biofilm formation on resin- of resin-based composites with low SR and SFE and high hydrophobicity (Burgers et al., 2010, Carlen et al., 2001) based composites have focused on the

development of materials featuring unfavourable conditions for the adhesion and colonisation of oral microorganisms, and included the development.

Resin-based composites are complex materials consisting of a hydrophobic resin matrix and less hydrophobic filler particles, which implies that a resin-based composite surface is never a homogeneous interface, producing matrix-rich and filler-poor areas, as well as matrix-poor and filler-rich areas, which accounts for the topographical and chemical differences within a single resin-based composite surface. For commercially available resin-based composites, differences in resin and filler chemistry, as well as in filler size and shape, account for differences in terms of surface roughness and surface chemistry after polishing (Carlen et al., 2001, Jefferies and Boston, 2010, Marghalani, 2010). In addition, the polishing procedure substantially modifies the physicochemical characteristics of a resin-based composite surface by removing the matrix-rich superficial resin based composite layers and producing a surface that is chemically and physically distinct from its unpolished counterpart (de Oliveira et al., 2012). Thus, differences in the composition of the resin-based composite, as well as the polishing procedure, might have an impact on biofilm formation on the resin-based composite surface (Ono et al., 2007, Takahashi et al., 2004).

Significantly less *S. mutans* biofilm formation was observed on polished resin-based composites than on unpolished resin- based composites during a four-day incubation (Ionescu et al., 2012), indicating that the proportions of resin matrix and filler particles on the surface strongly influence *S. mutans* biofilm formation using a modification of a commercially available Drip Flow Reactor (DFR 110; BioSurface Technologies, Bozeman, MT, USA). The modified design allowed the placement of customised specimen trays on the bottom of the flow cells and the complete immersion of the resin-based composite surfaces into the surrounding flowing medium.

Also the quantity of retained *S. mutans* biofilm was significantly less on the surface of diamond paste polished resin composite than on a regular silicon paper polished surface in the same *in vitro* system (Ono et al., 2007). *S. mutans* outgrowth was accelerated following direct contact with the surface of an aged resin composite without affecting the micro-hardness of the composite (Beyth et al., 2008).

A similar study showed an increase in surface roughness of restorative resin after one month's exposure to *S. mutans* biofilms (Fucio et al., 2008).

Although the polishing procedure will render composite surface less attractive for bacterial adhesion and colonisation, oral biofilm colonisation to polished surface will increase roughness and decrease micro-hardness which will deteriorate the material faster, thus polishing and smoothening of restorative materials is important but what is more important is enhancing the antibacterial activity of materials.

2.1.3.4.2 Resin Component and Biofilm Formation

Composite material showed more susceptibility to adhesion and colonisation by *Streptococci* biofilm than other dental materials suggesting that the exposure of composite resins may enhance the growth of cariogenic bacteria (Passariello & Gigola, 2013) (Hansel et al., 1998).

The enhanced growth of bacteria may be because the conversion of resin composites was not completed, approximately 30% to 50% of unpolymerised monomer was detected using Fourier transform infrared spectroscopy (FTIR) (Abed et al., 2015). It has been suggested that the release of ethylene- glycol dimethylacrylate and triethyleneglycol dimethacrylate from composite resins contribute to the growth of cariogenic bacteria like *Streptococcus sobrinus* and *Lactobacillus acidophilus* (Busscher et al., 2010, Schmalz et al., 2004).

In another study where they test the effect of monomers the results showed that resin composite may enhance the glucosyltransferase activity of the bacteria (Kawai and Tsuchitani, 2000); another investigation found that triethyleneglycol modulates the expression levels of glucosyltransferase B involved in biofilm formation (Khalichi et al., 2009).

2.1.3.5 Glass Ionomer

The setting reaction of glass-ionomer cements is an acid-base reaction between fluoroaminosilicate glass particles and a polyacrylic acid solution, the yielding structure is dimensionally more stable than composites. The glass-ionomer cements have the ability to adhere to tooth structure so potentially reduces microleakage and the enhanced fluoride release which has a potential impact on oral biofilm formation. Fluoride release occurs through an initially high burst release that may be between 1.6 and 1.8 $\mu g/mm^2$ during the first 24Hrs after which a sustained release follows (Wiegand et al., 2007).

Fluoride works as a buffer to neutralise acids produced by bacteria (Nicholson et al., 2000) and reduce the growth of caries-related oral bacteria (Nakajo et al., 2009), fluoride role in caries prevention will be discussed in section (2.3.1.1.1).

Glass-ionomer cement indeed collected a thin biofilm with a low viability (2% to 3 %), possibly as a result of fluoride release (Auschill et al., 2002). Levels of Streptococci in particular, including *S. mutans* (Seppa et al., 1995) and *S. sanguinis* (Hengtrakool et al., 2006), appeared reduced. However, an *in vitro* study (Al-Naimi et al., 2008) also showed that glass-ionomer cements containing fluoride did not reduce the amount of bacterial growth and biofilm formation on the surfaces bathed in saliva. This led to an assumption that either fluoride is not the main factor in controlling biofilm formation, or it was at low effective concentration, i.e. fluoride was diluted in the experimental fluids used. However, in the oral cavity, fluoride is subjected to washing out by continuous flow of

natural saliva (Wiegand et al., 2007) that makes the build-up of an effective fluoride concentration difficult.

2.1.3.6 Metallic Alloys

The adhesion of oral biofilm on metallic dental material surfaces (e.g. gold, amalgam, titanium and zirconia) has been reported both *in vivo* (Auschill et al., 2002, Burgers et al., 2010, do Nascimento et al., 2013) and *in vitro* (Lyttle and Bowden, 1993, Schmidlin et al., 2013) studies.

Biofilms on gold and amalgam are thick, but barely viable. It has been stated that mercury liberated from fresh amalgam alloy reduced some metabolic activities of plaque biofilms *in vitro*, but it did not affect biofilm formation (Lyttle and Bowden, 1993). The thickness of biofilm formation on amalgam reached 17 μm in five days.

It has been found that 98% of the mercury-resistant bacterial strains isolated from oral cavity were Streptococci, with *S. mitis* predominating (Ready et al., 2003) . Even though, plaque growing in the presence of amalgam could assimilate mercury, and biofilms appeared to facilitate mercury release *in vivo*. Aged amalgam with an undisturbed passive tarnish layer appeared unaffected by biofilm and did not liberate mercury (Lyttle and Bowden, 1993). Biofilm with extremely low viability was found on gold, probably due to its inert property which is unfavourable to the biofilm growth or specific binding sites of bacteria to gold surfaces (Auschill et al., 2002).

2.2 Saliva

Saliva is a clear, slightly acidic mucoserous exocrine secretion. Whole saliva is a complex mix of fluids from major and minor salivary glands and from gingival crevicular fluid, which contains oral bacteria and food debris (Edgar, 1992).

The average daily flow of whole saliva varies in health between 1 and 1.5 L. Percentage contributions of the different salivary glands during unstimulated flow are as follows: 20% from parotid, 65% from submandibular, 7% to 8% from sublingual, and less than 10% from numerous minor glands. Stimulated high flow rates drastically change percentage contributions from each gland, with the parotid contributing more than 50% of total salivary secretions (Edgar, 1990).

2.2.1 Composition

Saliva is composed of a variety of electrolytes, including sodium, potassium, calcium, magnesium, bicarbonate, and phosphates. Also found in saliva are immunoglobulins, proteins, enzymes, mucins, and nitrogenous products, such as urea and ammonia. These components interact in related function in the following general areas:

- Bicarbonates, phosphates, and urea act to modulate pH and the buffering capacity of saliva.
- Macromolecule proteins and mucins serve to cleanse, aggregate, and/or attach oral microorganisms and contribute to dental plaque metabolism.
- Calcium, phosphate, and proteins work together as an anti-solubility factor and modulate de-mineralisation and re-mineralisation.
- Immunoglobulins, proteins, and enzymes provide antibacterial action.

The components listed above generally occur in small amounts, varying with changes in flow, yet they continually provide an array of important functions. It is important to stress

that saliva, as a unique biologic fluid, must be considered as a whole that is greater than the sum of its parts (Dowd, 1999).

Salivary components, particularly proteins, are multifunctional (performing more than one function), redundant (performing similar functions but to different extents), and amphifunctional (acting both for and against the host) (Levine, 1993). Recent research into the complex roles of salivary proteins and mucins support this theory; it is summarised in the functions section (2.2.3) (Edgar et al., 2012).

Saliva is a very dilute fluid, composed of more than 99% water. Saliva is not considered an ultrafiltrate of plasma (Humphrey and Williamson, 2001). Initially, saliva is isotonic, as it is formed in the acini, but it becomes hypotonic as it travels through the duct network. The hypotonicity of unstimulated saliva allows the taste buds to perceive different tastes without being masked by normal plasma sodium levels. Hypotonicity, especially during low flow periods, also allows for expansion and hydration of mucin glycoproteins, which protectively blanket tissues of the mouth. Lower levels of glucose, bicarbonate, and urea in unstimulated saliva augment the hypotonic environment to enhance taste (Dawes et al., 2015).

The normal pH of saliva is 6 to 7, meaning that it is slightly acidic. The pH in salivary flow can range from 5.3 (low flow) to 7.8 (peak flow). Major salivary glands contribute most of the secretion volume and electrolyte and content to saliva, whereas minor salivary glands contribute little secretion volume and most of the blood-group substances (Edgar, 1990).

2.2.2 Flow

Several studies have reported that the mean flow rate of unstimulated/resting whole saliva in healthy persons during the day is in the range of 0.3–0.4 mL/min but with a large standard deviation (Heintze et al., 1983).

Salivary flow rate shows a circadian rhythm of high amplitude with peak flow in the late afternoon, while the flow rate is extremely low during sleep, which reduces the need to swallow during that time (Dawes, 1974). The total volume of saliva secreted per day has been estimated to be about 0.6 L (Heintze et al., 1983).

2.2.3 Function

Salivary function can be organised into five major categories that serve to maintain oral health and create an appropriate ecologic balance:

2.2.3.1 Lubrication and Protection

As a seromucous coating, saliva lubricates and protects oral tissues, acting as a barrier against irritants. These irritants include, but are not limited to, proteolytic and hydrolytic enzymes produced in plaque, potential carcinogens from smoking and exogenous chemicals, and desiccation from mouth breathing (Dawes et al., 2015). The best lubricating components of saliva are mucins that are excreted predominantly from minor salivary glands. Mucins are complex protein molecules that are present predominantly in two types MUC5B and MUC7, they are formed by polypeptide chains that stick together (Tabak, 1995). These mucins have the properties of low solubility, high viscosity, high elasticity, and strong adhesiveness and they are part of the acquired enamel pellicle (Gibbins et al., 2014, Siqueira et al., 2012b, Siqueira et al., 2007). Any intraoral contact between soft tissues, between soft tissues and teeth, or between soft tissues and prostheses benefits from the lubricating capability of saliva supplied largely by these mucins.

Mastication, speech, and swallowing all are aided by the lubricating effects of mucins (Iontcheva et al., 1997, Levine et al., 1987).

2.2.3.2 Buffering Action and Clearance

Buffering action and clearance are a second function of saliva through the following components: bicarbonate, phosphate, urea, and amphoteric proteins and enzymes. Bicarbonate is the most important buffering system. It diffuses into plaque and acts as a buffer by neutralising acids. Moreover, it generates ammonia to form amines, which also serve as a buffer by neutralising acids (Mandel, 1989a). More than 90% of the bicarbonate buffering ability of saliva is attributed to low-molecular-weight, histidine-rich peptides (Mandel, 1989b). Urea, another buffer present in saliva, releases ammonia after being metabolised by plaque and thus increases plaque pH (Dibdin and Dawes, 1998). The buffering action of saliva works more efficiently during stimulated high flow rates but is almost ineffective during periods of low flow with unstimulated saliva (Edgar, 1990, Garrett, 1987). Phosphate is likely to be important as a buffer only during unstimulated flow (Lagerlof and Oliveby, 1994). The pH of saliva may not be as important a measure for buffering action on caries as the pH of plaque, which saliva modifies (Edgar, 1976). Remaining fermentable carbohydrates and the buffering capacity of saliva affect plaque pH, unless the pH of the plaque is too low for bacterial enzymes to function. The resting pH of plaque (that is, the pH of plaque 2 to 2.5 hours after the last intake of exogenous carbohydrates) is 6 to 7.3 (Stephan, 1944). The pH rises during the first 5 minutes after the intake of most foods. The pH then falls to its lowest level, to 6.1 or lower, approximately 15 minutes after food consumption. Unless there is additional ingestion of fermentable carbohydrates, the pH of plaque gradually returns to its resting pH of 6 to 7.38-40. Thus, salivary buffering, clearance, and flow rate work in concert to influence intraoral pH (Bibby et al., 1986).

2.2.3.3 Maintenance of Tooth Integrity

Maintaining tooth integrity is a third function of saliva, one that facilitates the de-mineralisation and re-mineralisation process. De-mineralisation occurs when acids diffuse through plaque and the pellicle into the liquid phase of enamel between enamel crystals. Resulting crystalline dissolution occurs at a pH of 5 to 5.5, which is the critical pH range for the development of caries (Humphrey and Williamson, 2001). Dissolved minerals subsequently diffuse out of the tooth structure and into the saliva surrounding the tooth. The buffering capacity of saliva greatly influences the pH of plaque surrounding the enamel, thereby inhibiting caries progression (Stephan, 1944). Plaque thickness and the number of bacteria present determine the effectiveness of salivary buffers. Re-mineralisation is the process of replacing lost minerals through the organic matrix of the enamel to the crystals. Supersaturation of minerals in saliva is critical to this process. The high salivary concentrations of calcium and phosphate, which are maintained by salivary proteins, may account for the maturation and re-mineralisation of enamel (ten Cate, 1996).

Statherin, a salivary peptide, contributes to the stabilisation of calcium and phosphate salts solution, serves as a lubricant to protect the tooth from wear, and may initiate the formation of the protective pellicle by binding to hydroxyapatite (Gibbins et al., 2014, Siqueira et al., 2012b). Proteins in the protective pellicle, such as statherin, histatins, cystatins, and proline-rich proteins, are too large to penetrate enamel pores. Therefore, they remain on the surface, bound to hydroxyapatite, to aid in controlling crystalline growth of the enamel by allowing the penetration of minerals into the enamel for re-mineralisation and by limiting mineral egress (Dowd, 1999). This control of precipitation and mineral egress enhances the stability of hydroxyapatite in the outer tooth structure (Richardson et al., 1993). Low-molecular-weight protein fractions, thought to be derived from the proteolytic processing of larger proteins, are likely to be in exchange with dental

plaque fluid. These protein fractions help adjust and augment re-mineralisation, microbial attachment, and plaque metabolism at the tooth-saliva interface (Perinpanayagam et al., 1995).

The presence of fluoride in saliva speeds up crystal precipitation, forming a fluorapatite-like coating that is more resistant to caries than the original tooth structure. In that sense, small amounts of de-mineralisation have been suggested as advantageous for the tooth because enamel components of magnesium and carbonate are replaced with the stronger, more caries-resistant fluorapatite crystals (Edgar, 1990). Fluoride in salivary solution works to inhibit dissolution of apatite crystals. The contribution of saliva to the de-mineralisation re-mineralisation process points to the importance of monitoring salivary flow, especially in patients taking multiple medications or having systemic entities that decrease salivary flow. For patients with exposed root surfaces or with recurrent or incipient carious lesions, fluoride supplementation can promote re-mineralisation. Salivary stimulants and substitutes also should be encouraged for patients with salivary hypofunction.

2.2.3.4 Antibacterial Activity

Saliva contains a large number of proteins and peptides which have been shown to have anti-bacterial, anti-viral and anti-fungal effects (Fábián et al., 2012, Gorr, 2009, Helmerhorst, 2012, Malamud et al., 2011, van 't Hof et al., 2014). However, since the mouth is known to contain more than 1000 different species of microorganisms, it is clear that these antimicrobial factors are not present in sufficient concentration to eliminate the members of the normal flora. For instance, α-amylase is a growth inhibitor of *P. gingivalis*, a periodontal pathogenic bacterium (Ochiai et al., 2014), but this organism still survives in the mouth. It is possible though that antibacterial factors, in conjunction with good oral hygiene, function to maintain the proportion of the harmful oral flora at

levels sufficiently low for oral health to be maintained. In addition, they may maintain the oral flora at sufficiently low levels that systemic infection with oral microorganisms rarely occurs. They may, of course, prevent some pathogenic microorganisms from ever colonising the mouth at all. There are several cationic proteins in saliva including histatin, statherin and alpha and beta defences. Statherin's primary function may be to inhibit the crystallisation of calcium phosphate from saliva but it also inhibits the growth of anaerobic bacteria (Gorr, 2009).

Lactoferrin is a chelator which has high affinity for iron (Fe^{3+}) and the removal of this essential metal inhibits the metabolic activity of several pathogenic microorganisms. Lysozyme is derived from the salivary glands, crevicular fluid and salivary leukocytes. It is strongly cationic and damages microbial cell walls by hydrolysing the ß(1–4) bond between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan layer of the bacterial cell wall (Oppenheim et al., 1988).

The main antibody to oral microorganisms in saliva is secretory IgA (sIgA), the functional significance of sIgA is uncertain, as persons with a hereditary lack of IgA do not appear to be more susceptible to oral disease, although they may show an increase in IgM in their saliva (Helmerhorst, 2012). In addition, small amounts of IgG and IgM may enter whole saliva via the gingival crevices.

2.2.3.5 Taste and Digestion

Saliva plays a critical role by providing the fluid in which solid materials may dissolve and in distributing the material around the oral cavity to the locations of the taste buds (Matsuo, 2000);(Deshpande et al., 2010).

The hypotonicity of saliva enhances the tasting capacity of salty foods and nutrient sources. This enhanced tasting capability depends on the presence of protein and gustin, which bind zinc (Moss, 1995). Saliva has an early, limited role in total digestion by

beginning the breakdown of starch with amylase, a major component of parotid saliva that initially dissolves sugar (Woolnough et al., 2010). The contribution of saliva to starch breakdown is limited because most of the digestion of starch results from pancreatic amylase, not salivary amylase. Salivary enzymes also initiate fat digestion (Valdez and Fox, 1991). More importantly, saliva serves to lubricate the food bolus, which aids in swallowing. When one considers the contribution of saliva to taste and early digestion, it becomes clear that artificial supplements would be difficult to develop.

2.3 Prevention Strategies of Plaque-Induced Oral Diseases

Traditionally prevention of dental caries and the periodontal diseases is targeted at the mechanical or non-specific control of the precipitating factor (dental plaque). On the other hand, the individual host response and other confounding factors can affect disease initiation and progression. Using approaches that could modulate or prevent oral biofilm formation will be a valuable complement to mechanical plaque control.

Hence, a critical approach is the development of new materials that restrict biofilm formation and enhance the control of oral disease.

Ideally dental materials with multiple anti-biofilm properties would be available, and could include;

- Inhibition of initial binding.
- Preventing biofilm growth.
- Affecting microbial metabolism in the biofilm.
- Killing biofilm bacteria.
- Detaching biofilm.
- Modifying the acquired pellicle to attract non cariogenic microorganisms.

The ultimate goal of the development of dental materials with anti-biofilm properties is to reduce disease occurrence and increase the success rate of dental treatments. Significant efforts have been made to study the characteristics of biofilm accumulation and adhesion, modification of dental material composition and the release of antimicrobial compounds.

Ideally such strategies should prevent plaque biofilm formation without affecting the biological equilibrium within the oral cavity.

2.3.1 Approaches and Mechanisms of Anti-Biofilm Systems of Dental Materials

Depending on the mechanisms of action, the approaches used are classified to;

2.3.1.1 Interference with Bacterial Attachment and Metabolism

Embedding antimicrobial ions is the most common strategy to the dental materials to have anti-biofilm properties, multiple ions were included however the main ones are summarised in the following.

2.3.1.1.1 Fluoride

The mechanisms of fluoride interference with bacterial metabolism and dental plaque acidogenicity include the inhibition of the glycolytic enzyme enolase and the proton-extruding ATPase as well as the bacterial colonisation and competition. Furthermore, intracellular or plaque associated enzymes such as acid phosphatase, pyrophosphatase, peroxidase and catalase may be affected by fluoride ions (Hamilton, 1990).

Conflicting data resulted from *in vitro* studies on fluoride effect, some of them showed that low fluoride levels reduced bacterial growth, dental plaque composition and acid production (Friedl et al., 1997, Kan et al., 1997, Seppa et al., 1993, Seppa et al., 1995). However, fluoride concentrations needed to have an antimicrobial effect is mostly surpass the concentration needed to reduce the solubility of apatite i.e. only small amounts of fluoride (approximately 0.03–0.08 ppm) in re-mineralising solutions are necessary to shift the equilibrium from de-mineralisation to re-mineralisation . Therefore, it is often considered that the antimicrobial effect of fluoride is less important when the fluoride concentration required for inhibition of de-mineralisation is exceeded.

Nevertheless, several clinical studies found that the fluoride concentration of plaque on or adjacent to glass-ionomers is increased and the proportion of *S. mutans* in plaque is reduced (Benelli et al., 1993, Berg et al., 1990, Forss et al., 1991, Moura et al., 2004,

Svanberg et al., 1990a, Svanberg et al., 1990b). Contradictory, another studies (Seppa et al., 1992) found that fluoride concentration of approximal plaque of teeth close to glass ionomer restorations wasn't increased either after two or four weeks.

However, it was shown that fluoride can inhibit the growth of oral streptococci *in vitro* at concentrations of 0.16–0.31 mol/l (Maltz and Emilson, 1982). This concentration is much higher than found in dental plaque adjacent to fluoride-containing restoratives. Due to this fact, the results of some *in vivo* investigations mostly showed that the fluoride concentrations released from one- to three-year-old restoratives are not high enough to affect the metabolism of caries- associated bacteria (Forss et al., 1995, van Dijken et al., 1991, van Dijken et al., 1997). In contrast, studies investigating glass-ionomer restorations up to one month after insertion showed a correlation between fluoride release and reduced *S. mutans* counts in plaque (Benelli et al., 1993, Berg et al., 1990, Forss et al., 1995, Svanberg et al., 1990a, Svanberg et al., 1990b) or saliva (Koch and Hatibovic-Kofman, 1990). However, levels of *S. mutans* in plaque from composite or amalgam fillings were lower than in plaque formed on glass ionomers (Berg et al., 1990, Forss et al., 1995, Svanberg et al., 1990a, Svanberg et al., 1990b).

Also fluoride present in resin-modified glass ionomers has been related to decrease in microbial colonisation of carious dentine. *In vivo*, a resin-modified glass ionomer restoration led to a larger decrease in counts of *S. mutans* and *lactobacilli* in remaining carious dentine than amalgam after 6 months (Kreulen et al., 1997, Weerheijm et al., 1999). In contrast, Weerheijm et al. (Weerheijm et al., 1993) found no differences in numbers of oral *streptococci* and *lactobacilli* in dentin lesion two years after insertion of a glass ionomer or a resin-based sealant.

Alongside fluoride, other components simultaneously released from ionomeric-based (e.g. aluminium and zinc) or resin-based materials (monomers and catalysts) may be

involved in the antibacterial activity of the materials (Hayacibara et al., 2004, Imazato, 2003, Moura et al., 2004).

In conclusion, fluoride is known to inhibit the biosynthetic metabolism of bacteria, but these antimicrobial effects on caries prevention are often regarded as little or of no importance as compared to the direct interactions with the hard tissue during caries development and progression i.e. de-mineralisation re-mineralisation process. There is still a lack in understanding whether the antibacterial effects of fluoride clinically contribute to the anti-caries effect and whether fluoride leached from restorative materials into plaque or saliva may be relevant for these antibacterial effects.

2.3.1.1.2 Silver

Silver has been used for diverse dental applications ranging from silver based restorative material i.e. amalgam fillings (Neelakantan et al., 2012) to silver coated implants (De Giglio et al., 2013). Silver ions released from dental materials in aqueous solution can attach to the bacterial membrane and penetrate biofilm, which causes bacterial inactivation and prevents bacterial replication by binding to microbial DNA and to the sulfhydryl groups of the metabolic enzymes in the bacterial electron transport chain (Darouiche, 1999, Liao et al., 2010).

In this project silver complexes were an area of interest and their use in clinical dentistry is discussed in section (2.5).

2.3.1.1.3 Zinc oxide (ZnO)

By releasing oxygen and zinc ions, ZnO demonstrates antibiofilm properties, it has been shown that active oxygen can inhibit the growth of planktonic microbes (Aydin Sevinc and Hanley, 2010). Another potential antimicrobial mechanism of ZnO occurs through the leaching of zinc ions into the growth medium (Aydin Sevinc and Hanley, 2010), inhibiting the active transport and metabolism of sugars as well as disrupting enzyme

systems of dental biofilms by exchanging magnesium ions essential for enzymatic activity of the plaque (Devulapalle and Mooser, 1994). Zinc can also reduce acid production by *S. mutans* biofilms due to its ability to inhibit glucosyltransferase activity (He et al., 2002, Wunder and Bowen, 1999).

2.3.1.1.4 Quaternary Ammonium Compounds (QAC)

Primarily these compounds work through the release of cationic monomers (Li et al., 2009). Cationic monomers have been reported to suppress the expression of glucosyltransferase enzyme genes (*gtf*) which is responsible for the production of glucan and subsequently EPS formation, that might end in directly affecting plaque biofilm accumulation and adherence by reducing the amounts of biofilm-building enzymes, therefore decreasing EPS formation and bacterial adherence (Busscher et al., 2010). Since the deposition of EPS and the attachment of bacteria are compromised, the accumulation of biofilm proposed to be reduced (Ooshima et al., 2001).

2.3.1.1.5 Chlorhexidine (CHX)

Chlorhexidine has been incorporated into restorative materials and titanium implant coatings to reduce dental biofilm formation (Hu et al., 2013, Verraedt et al., 2011). The CHX molecule reacts with negatively charged groups on the cell surface, causing an irreversible loss of cytoplasmic constituents, membrane damage, and enzyme inhibition (Shen et al., 2011). It is likely that ionic interactions occur between the positively charged CHX molecules and the negatively charged extracellular matrix of the biofilm. At sub-lethal concentrations, chlorhexidine can interfere with the metabolism of oral biofilm by inhibiting sugar transport, acid production, and various membrane functions in Streptococci.

2.3.1.2 Bio-mineralisation Approach

In the ideal circumstances an antimicrobial dental material would combine high minerals delivery, high pH, and a high alkaline buffer capacity which in turn favours re-mineralisation of carious enamel.

Bio-ceramic dental materials cause calcium silicates/phosphates release which enhance bio-mineralisation (Waltimo et al., 2007). Moisture from the environment promotes the hydration to produce calcium silicate hydrogel and calcium hydroxide to elevate the pH (Zhang et al., 2009). Silica dissolved in a high-pH environment may directly inhibit bacterial viability (Zehnder et al., 2006). Calcium hydroxide subsequently reacts with the phosphate to form hydroxyapatite and water. This water may participate in the reaction cycle again to produce more calcium silicate hydrogel and calcium hydroxide, the continuous diffusion of calcium hydroxide that results is meant to be responsible for the killing of oral bacteria. (Yang et al., 2002).

2.3.2 Dental Restorative Materials with Enhanced Anti-Biofilm Formation Properties

2.3.2.1 Composite

Research focused on preventing biofilm formation and enhanced remineralisation of the surrounding enamel, has led to enhanced fluoride-releasing composites being developed. This was achieved through the incorporation of water-soluble salts, fluoride-releasing filler systems, or matrix-bound fluoride (Wiegand et al., 2007). Fluoride release requires penetration of water into the polymer matrix, which can be stimulated by increasing matrix hydrophilicity through the introduction of 2-hydroxyethyl methacrylate (Chan et al., 2006). The amount of fluoride released from composites is lower than that from glass-ionomer cement and decays over time (Yap et al., 1999). However, clinical studies have reported conflicting data as to whether fluoride-releasing materials significantly prevent or inhibit secondary caries and affect the growth of caries-associated bacteria compared with non-fluoridated restoratives (Wiegand et al., 2007). As is often the case with antimicrobial release systems, these conflicting results may be due to experimental differences in area of the releasing material versus the fluid volume into which the antimicrobial is released and possible wash-out.

Silver has been added to composite using phosphate-based glasses loaded with silver and found to reduce the viability of 300-µm-thick adherent biofilms of *S. sanguinis* (Mulligan et al., 2003).

Killing by direct contact rather than releasing anti-bacterial substances is considered to be a more promising approach. For that reason, resin composites have been modified by the addition of antibacterial components, such as 12-methacryloyloxydodecylpyridinium bromide combined with quaternary ammonium and a methacryl group in the resin matrix. This material inactivated bacteria upon coming into contact with its surface, hence

inhibiting biofilm formation on the composite surface (Imazato and McCabe, 1994; Imazato et al., 1994, 1999), even after contact with saliva (Ebi et al., 2001). Whether these modifications can result in clinically significant inhibition under the dynamic oral cavity conditions remains to be seen (Imazato, 2003).

2.3.2.2 Glass Ionomer

Beside the presence and release of fluoride which has been discussed earlier, most of the data were from *in vitro* studies (Wiegand et al., 2007). However, clinical studies found that secondary caries was still the main reason for GIC failure (Manhart et al., 2002, Deligeorgi et al., 2001, Mjor et al., 2002, Forss and Widstrom, 2004), indicating that the fluoride-release from GICs is not enough to inhibit bacterial growth or combat tooth destruction by the bacteria (Forss and Widstrom, 2004).

Numerous antibacterial agents have been incorporated into GIC to improve its antibacterial properties with a focus on the release approach, e.g. zinc ions (Osinaga et al., 2003), furanone which is a heterocyclic organic compound known also as γ-crotonolactone (GCL) (Weng et al., 2012), and iodine and CHX (Takahashi et al., 2006).

The incorporation of CHX into GICs has resulted in excellent antibacterial effect *in vitro* (Tuzuner et al., 2011). High precision visualisation by Confocal microscopic analysis revealed that bacterial viability of the plaque biofilm on CHX containing GIC was significantly lower than that on GIC alone (Du et al., 2012). Propolis (resinous mixture produced naturally by honey bees) addition to GIC exhibited inhibition zones and their dry biofilm weights were less than the pure GIC (Topcuoglu et al., 2012). With the addition of epigallocatechin-3- gallate (EGCG) the antibiofilm properties of *S. mutans* were enhanced (Hu et al., 2013).

Metal reinforced glass ionomer cements (e.g. Ketac Silver, Espe) which contain silver-tin alloy fibres or flakes being sintered to the glass particles (Mount, 1998; Nicholson, 1997) were developed to improve the mechanical properties of the cements and increase their wear resistance rather than their antibacterial activity (Meffert, 1996; Walls, 1987; Williams J A, 1992). Due to the amalgam-like optical performance, metal-reinforced materials showed an enormous commercial success and were consequently used as posterior materials for the primary dentition as well (Krämer N, 1996).

2.3.2.3 Implants

Since the concept of “the race for the surface” was launched in 1987 (Gristina, 1987), describing the fate of an infected implant as a race for the surface between tissue integration and biofilm formation, preventive measures have aimed primarily at discouraging biofilm formation (Busscher et al., 2010). Attempts to enhance implant surface to be Infection-resistant may be constructed by various approaches. Some of the most studied methods are: finishing implant material surfaces with repellent coatings, antimicrobials, surfactants, and hydroxyapatites (Arciola et al., 2012, Campoccia et al., 2013, Petrini et al., 2006).

2.4 *In Vitro* Model Systems to Study Oral Biofilms

Generally oral biofilm models, are commonly used to help us understand complex processes and the factors affecting the oral diseases. They help us to accurately predict, in a controlled and simplified way, a clinical outcome which can lead us to preventive actions for a disease (Featherstone, 1996).

When using a model, it is important to consider the research question in order to carefully evaluate which model type should be used so that results are interpreted correctly. The complexity of biofilm research requires different approaches to address various questions. First, the development of *in vitro* models should be based on prior knowledge of the *in vivo* situation. Then, as our understanding of the oral cavity progresses, model systems can be improved. There are many interactions and processes between bacteria in the biofilm, those interaction can be varied depending on bacterial species and prevalent external conditions factors which may complicate the interpretation of findings (Blanc et al., 2014). Even though a model cannot capture all of the details involved with caries formation, it can give us a means of performing reproducible experiments under controlled conditions. Obviously there are ethical limitations with *in vivo* studies in relation to caries and periodontal diseases. Therefore, different *in vitro* techniques have been developed and are continuously improved to better address the study question, to help interpret the results and to obtain as much information as possible with other than clinical testing (Blanc et al., 2014).

Models can be divided into two groups:

- **Closed Batch Culture.**
- **Open Continuous Culture**; can be further divided into
    - **Artificial Mouth Models (AMM).**
    - **Flow Cells.**

Batch and continuous culture methods are used to grow a monoculture biofilm, a defined consortium biofilm (from two up to ten species) or a microcosm biofilm (using saliva or plaque sample as inoculum) (Coenye and Nelis, 2010). The different bacterial biofilm models were used to study the origins of caries, caries prevention, how cariogenicity changes with different bacteria and how diet or other compounds and materials affect cariogenicity (Coenye and Nelis, 2010). Furthermore, these models were used to study the periodontal diseases and its controlling factors (Park et al., 2014).

2.4.1 Batch Biofilm Models

In these models a biofilm is formed either on a plate wall, on the surface of disks, coupons or pegs or on human or bovine enamel within the well. It is a closed system so that the environment inside the well changes during the test as nutrients are consumed and metabolic products accumulate unless the growth media are replaced (Coenye and Nelis, 2010). The frequency for changing the growth media depends on the model set up.

The drawback for these models is they are lacking of the flow fluids and nutrients which is not the case with saliva in the oral cavity. Although some models have been adapted to create a liquid shear force by dipping the biofilms in saline or other liquid during biofilm formation (Guggenheim et al., 2004), they still represent relatively simple environments that can be reproduced with minimal set up time. The most significant advantages of batch models are their ability to offer means of comparing multiple test compounds or

conditions simultaneously; they only require small amounts of reagents and are convenient, reproducible, and economical to use (Coenye and Nelis, 2010).

One of the most commonly used batch biofilm models is the Zürich biofilm model which uses six microbial species (*S. oralis, S. sobrinus, A. naeslundii, V. dispar, F. nucleatum, and Candida albicans*);(Guggenheim et al., 2004). Using fluorescently labelled antibodies and confocal laser scanning microscopy (CLSM), this model allows the interspecies associations to be studied with respect to biofilm formation and how macromolecules of different sizes can penetrate the biofilm *in vitro*. This model and its variants have been used extensively to evaluate the effect of different substances in the biofilm formation process (e.g. plant extracts (Furiga et al., 2014), chlorhexidine (Guggenheim and Meier, 2011), and xylitol (Giertsen et al., 2011). Furthermore, the model has been used to study the effect of oral probiotics on a growing biofilm (Marttinen et al., 2013), as well as de- and re-mineralisation (Guggenheim et al., 2004, Arthur et al., 2013) in a biofilm with variable formation times. It has also been used for developing methods to analyse biofilm microbes (Alvarez et al., 2013). For example, (Marttinen et al., 2012) used a variation of this model incorporating three species of bacteria (*S. mutans, S. sanguinis, and A. naeslundii*) to study the effect of xylitol in a young biofilm. Another modification of this model is the three-species version (*S. mutans, S. oralis, and A. naeslundii)* developed to mimic ecological changes with respect to cariogenic biofilm formation and to investigate the relationship between *S. mutans* and exopolysaccharides (Koo et al., 2010).

The Calgary Biofilm Device (a 96-well plate system using lids with 96 pegs for biofilm formation) was developed in 1999 (Ceri et al., 1999). This model allows rapid testing for antibiotic susceptibility in a biofilm model, with or without agitation. As biofilm growth differs in comparison to planktonic growth, it was important to develop a means of testing

susceptibility of the bacteria within biofilm to antimicrobials. In a Calgary device, inhibitory concentrations can be analysed by comparing the positive control to the lowest concentration of the antimicrobial with minimum 10% of difference in OD_{600}. In addition, biofilms can be visualised using scanning electronic microscopy (SEM) or CLSM (5). The method has been used extensively to determine the Minimal Biofilm Inhibitory Concentration, Minimal Biofilm Eradication Concentration and Biofilm Bactericidal Concentration for various antibiotics and antimicrobials, but mainly in non-caries related biofilm studies (Ceri et al., 1999, Macia et al., 2014).

A microcosm batch biofilm was grown in a polystyrene based coverslip with different media, and it was evaluated using Checkerboard DNA-DNA hybridisation analysis (Filoche et al., 2007). This model was used to assess responses to environmental factors such as changes in growth media, growth volume, and sucrose addition. It showed a behaviour similar to natural oral biofilm, and the model was able to illustrate individual responses to environmental changes. Van de Sande et. al. (van de Sande et al., 2013) developed a microcosm batch biofilm model for estimating demineralisation using bovine enamel discs, saliva analogue growth media, and periodical sucrose exposures. The model was used to compare mother-child pairs and their susceptibility to a regular sucrose exposure. It was seen that under sucrose exposure biofilms showed similar microbiological changes and mineral loss regardless of the individuals, thus suggesting that diet and behavioural factors can be more important causes of caries development than transmission of microbes (Azevedo et al., 2014).

2.4.2 Continuous Biofilm Models

2.4.2.1 Artificial Mouth Models (AMM)

The term artificial mouth model (AMM) is usually used to describe dental biofilm systems with a continuous, open-surface fluid flow rather than flow cells with closed flow

(Shu et al., 2000). The AMM provides intermittent or continuous flow of nutrients over the biofilm, mimicking the *in vivo* situation as closely as possible (Tang et al., 2003).

An AMM simulates oral conditions in terms of temperature, humidity, sucrose supply, pH, and nutrient (i.e. saliva flow rate), but still there are differences between different AMMs in biofilm formation time, nutrient media, and equipment used. As the equipment is more complex than in batch systems, AMMs usually have less replicates, but instead they offer a means to investigate the mechanism of action of microbes and the compounds being tested as well as the overall growth and structure of plaque. This is due to the controlled environment that more closely mimics the oral cavity *in vivo* (Mei et al., 2013).

(Tang et al., 2003) provide a review of the history, development, and structure of the AMM. In AMM both defined multispecies biofilm and microcosm has been used, a defined multispecies biofilm AMM allows for a more detailed and easier analysis of bacteria present in comparison to a microcosm AMM. An AMM with four-species (*S. mutans, S. sobrinus, A. naeslundii, and Lactobacillus rhamnosus*) has been used to study enamel and root caries and to compare single and multispecies models (Shu et al., 2000). Consortia biofilms were usually larger than monospecies biofilms, and they also tended to cause more enamel softening. The addition of sucrose to the consortia biofilm created a similar pH curve as that found in vivo.

A defined multispecies AMM with a different set of bacterial species (*S. mutans, S. sobrinus, Lactobacillus acidophilus, L. rhamnosus, and A. naeslundii*) was used to study the mechanism of action of silver diamine fluoride on the biofilm. It was found that it inhibits biofilm formation, and it also reduces demineralisation (Mei et al., 2013). The arrangement of bacteria within the biofilm was determined by CLSM.

A slightly modified AMM using a three-species (*S. mutans, S. sobrinus, and Streptococcus gordonii*) system was developed to evaluate the formation of secondary caries around restorations and to assess the effectiveness of bonding material (Hayati et al., 2011). For the formation of secondary caries, a biofilm was first grown in a continuous flow reactor for 20 Hrs on a saliva-coated specimen and subsequently incubated in a batch system for 7-30 days. The model produced caries lesions around composite resin restorations and the protective effect of the bonding system was verified.

Forssten et. al. (Forssten et al., 2010) presented a dental caries simulator consisting of a continuous flow system with standard artificial saliva flow (Bjorklund et al., 2011). The temperature was controlled, and the artificial saliva was continuously mixed. Hydroxyapatite (HA) disks were used as a model tooth and as an adhesive support for the bacteria. The system could be inoculated with single or multiple bacterial species and test substances could be added either continuously or in pulses during simulation. The system has 16 replicate vessels which enable parallel testing of multiple conditions (Forssten et al., 2010). With this model, it is possible to monitor the initial steps of bacterial adherence to the HA-discs and the subsequent biofilm formation. It can then be used to study the effects of various substances such as polyols on bacterial quantities and adherence.

Of all the *in vitro* models mentioned, microcosm AMM comes closest to replicating *in vivo* conditions in the oral cavity. However, as the complexity of bacteria increases also the interpretation of the results becomes more complicated. The advances in the methods used for analysing the biofilm and its components have led to a deeper understanding of the biofilm formation process and the factors connected to it. The microcosm AMM is a valuable tool for studying the function and structure of dental biofilm. The focus with microcosm AMM studies was initially on biofilm growth, metabolism (pH changes, the effect of sucrose, and growth media), and de-mineralisation and re-mineralisation

processes (Sissons, 1997, Sissons et al., 1998, Wong and Sissons, 2001). A further variation of the AMM is the microcosm constant depth film fermenter which has been used to study the effect of chlorhexidine and tetracycline on the microbiota composition in biofilm (Pratten et al., 1998, Pratten and Wilson, 1999, Ready et al., 2002). The structure and viability of the biofilm were found to be similar *in vivo* as judged by CLSM (Hope et al., 2002, Pratten et al., 2000).

Thirty-six bacterial species were also identified in the supragingival biofilm using a combination of culture and molecular methods (PCR) (Pratten et al., 2003). The method used to identify different microorganisms in a microcosm biofilm developed; that is, denaturing gradient gel electrophoresis (DGGE) allowed the individual variations and changes of the bacterial populations to be captured during the growth of the biofilm (Rasiah et al., 2005). More recent methods of detecting bacteria present in biofilm, such as qPCR and Human Oral Microbial Identification Microarray (HOMIM), have enabled more accurate analysis of the bacterial population composition (Rudney et al., 2012, Zaura et al., 2011). In addition, newly developed methods such as cross- polarisation optical coherence tomography (CP-OCT) enable the evaluation of the early stages of caries formation (Chen et al., 2012). CP-OCT allows visualisation of the biofilm without disturbing it. With this method, the sample is kept hydrated, and images are taken within minutes as the sample is removed from the biofilm reactor. Lately, microcosm biofilm models have increasingly been used for studying the possibilities of different restoration materials (e.g. dimethylaminododecyl methacrylate and nanoparticles of silver or calcium phosphate) to inhibit the formation of secondary caries (Chen et al., 2014, Li et al., 2014, Zhang et al., 2013).

2.4.2.2 Flow Cell Biofilm Models

In flow cells, the liquid phase moves only in one direction and mixing happens by diffusion; therefore, conditions vary at different sites within the reactor (Coenye and Nelis, 2010). Flow cells are especially useful for studying the development of biofilm formation and morphology. Sequential colonisation can be observed in real-time using microscopic analyses of undisturbed biofilms.(Hannig et al., 2010, Pamp et al., 2009) provide reviews of various staining and visualisation techniques that can be used with flow cell biofilms.

Development of 9 to 11-member-consortia biofilm systems by Marsh and colleagues was a major advance in biodiverse synthetic plaque models at that time (McKee *et al*, 1985). These systems contain representatives of major plaque species *S. sanguis*, *S. oralis*, *S. mutans, A. naeslundii*, *N. subflava, P. gingivalis*, *V. dispar, Prevotella nigrescens* and *F. nucleatum*, selected to allow for complete microflora analysis.

Consortium biofilms containing all species have been cultured on hydroxyapatite (HA) surfaces suspended in the chemostat and showed effects similar to those of glucose/pH pulsing (Marsh, 1994) . Conditioning the HA surface with saliva or glucosyltransferase preparations differentially affected both total plaque growth and individual species ((Marsh, 1994); Bradshaw et al, 1995). Initially, *N. subflava* predominated, but later, obligate anaerobes did so even if *N. subflava* was omitted. The anaerobes, even in an aerated planktonic phase, were protected from O_2 by co-aggregation with aerobic and facultative species, mediated by *F. nucleatum* for species pairs which did not otherwise co-aggregate (Bradshaw et al., 1996, 1997).

A four-species (*S. gordonii, A. naeslundii, Veillonella atypica, and F. nucleatum*) flow cell biofilm model was used to evaluate the mechanism of early biofilm formation. Biofilms were analysed using fluorescent stains and fluorescent in situ hybridisation

(FISH) probes visualised by CLSM (Foster and Kolenbrander, 2004). It was found that species inoculated sequentially had more biomass than coaggregate-inoculated biofilms and *S. gordonii* was a major component of the formed biofilm. Schlafer *et al* (Schlafer et al., 2011) presented a variation of the flow cell biofilm model which focuses on changes in the early caries process when only mildly acidogenic bacteria are present. This five-species (*S. oralis, S. sanguinis, S. mitis, Streptococcus downei, and A. naeslundii*) flow cell biofilm model (26 Hrs old biofilm) is highly reproducible and shows structural similarity to *in vivo* biofilms. The structure and composition of the biofilms were analysed using FISH with CLSM. In addition, the model also uses pH-sensitive ratio metric fluorescent dyes to evaluate pH- levels at the biofilm-substratum interface. The model can be useful for testing substances that affect early stages of caries development (Schlafer et al., 2012).

Blanc et al (Blanc et al., 2014) developed a six-species (*S. oralis, A. naeslundii, V. parvula, F. nucleatum, A. actinomycetemcomitans, and P. gingivalis*) biofilm model for evaluating biofilm development under flow and shear conditions that can be used to assess, for example, antimicrobial substances. Bacteria were first grown in a Lambda Minifor bioreactor, the bacterial suspension was transferred to a modified Robbins device with HA-discs pre-coated with saliva and biofilm was formed in 3-9 days. SEM and CLSM were used to study the composition of the biofilm during formation of the biofilm, and the amount of bacteria was determined by culturing.

Overall, these consortia systems embody a powerful synthetic approach for studying the behaviour of plaque biofilms. However, biofilm models are difficult to compare due to the differences in biofilm formation times, different growth media, and varying bacterial species used in different situations. But each model has advantages and disadvantages

which could be useful in the deciding which to use or improve depending on the purpose of the study, Table 2.1 summaries the advantages and disadvantages of each model.

By using simple equipment and techniques we could have more consistent results for multiple conditions using the batch biofilm model. Therefore, a batch biofilm model of 8 species consortium was chosen in this project.

Table 2.1 The main differences between batch biofilm model, artificial mouth model (AMM), and flow cell biofilm models**.**

	Batch	**Continuous**	
		AMM	**Flow cell**
Advantages	• Multiple compounds tested simultaneously. • Multiple conditions tested simultaneously. • Small volumes of reagent. • Easy to perform. • Simple equipment.	• Flow conditions. • Conditions closely mimicking in vivo situation. • Product and nutrient concentration stable during biofilm formation. • Perfect mixing	• Flow conditions. • Possibility to analyse biofilm formation real-time. • Intermediate complex equipment
Disadvantages	• Closed system. • No flow	• Requires larger volumes of reagents. • Only one condition/run can be tested. • Complex equipment. • Usually less replicates. • More expensive.	• Conditions vary at different sites in the reactor. • Only one condition/run can be tested. • Usually less replicates.

2.5 Silver in Dentistry

Silver and its compounds are known to have extraordinary bacteriostatic and bactericidal properties. The silver ion exhibits broad-spectrum biocidal activity toward many different bacteria, fungi, and viruses and is accepted to be the active component in silver-based antimicrobials (J. S. Kim et al., 2007; K. J. Kim et al., 2008; Pal, Tak, & Song, 2007; Panacek et al., 2006). It has been used in different fields in medicine, including wound dressings (Alt et al., 2004), catheters (Samuel & Guggenbichler, 2004), and prostheses (Gosheger et al., 2004). Besides being a potent antimicrobial, silver has many advantages, such as low toxicity and good biocompatibility with human cells (Hardes et al, 2007; Necula et al., 2012), long-term antibacterial activity, due to sustained ion release (Damm, Münstedt, & Rösch, 2008), and low bacterial resistance (Percival, Bowler, & Russell, 2005).

2.5.1 Forms of Incorporation

2.5.1.1 Silver Nano-particles (AgNPs)

Research has concentrated on nanometric silver particles (AgNPs) prepared by a variety of synthetic methods. Several formulations been shown to be effective antimicrobial agents (Alt et al., 2004, Aymonier et al., 2002, Baker et al., 2005, Melaiye et al., 2005, Sondi and Salopek-Sondi, 2004).

AgNPs have also been applied in several areas of dentistry, for example, endodontics (Afkhami et al., 2015, Samiei et al., 2013), dental prostheses (Nam, 2011), implants (Flores et al., 2010, Zhao et al., 2011), and restorative dentistry (Cheng et al., 2012a, Cheng et al., 2012b, Durner et al., 2011). AgNPs incorporation aims to avoid or at least to decrease the microbial colonisation over dental materials, increasing oral health levels and improving life quality.

2.5.1.1.1 Properties of AgNPs

Due to their small size which range from 5-100 nm, AgNPs possess chemical, physical, and biological properties distinctive from those presented by traditional bulk materials (Eckhardt et al., 2013). These smaller particles have a larger surface area and consequently provide potent antibacterial effects at a low filler level, and that may have less effect on the physical properties when added to restorative materials (Damm et al., 2008, Peulen and Wilkinson, 2011)

Another advantage provided by the small size is that they have the ability to penetrate through cell membranes more readily, resulting in higher antimicrobial activity, which is especially valuable for the treatment of biofilms that are more resistant to antimicrobial agents than planktonic pathogens (Chaloupka et al., 2010).

2.5.1.1.2 Chemical Routes of Synthesis

Many routes have been introduced for the synthesis of silver nanostructures, which can be categorised as chemical methods, physical and biological methods but chemical methods especially chemical reduction is the simplest and the mainly used method.

Chemical methods for the syntheses of silver nanostructures can be subdivided into chemical reduction, electrochemical techniques, irradiation-assisted chemical methods, and pyrolysis. The chemical synthesis of AgNPs in solution usually contains three main components; metal precursors, reducing agents, and stabilising/capping agents. Widely used reducing agents include borohydride, sodium citrate, ascorbic acid, alcohol, and hydrazine compound (Park et al., 2004);(Abou El-Nour et al., 2010). These methods are expected to result in a narrow particle size distribution and particles of uniform shape. Since the metal colloids tend to coagulate, they are usually unstable as a result, the sustainability of their antibacterial activities are poor. This problem can be greatly overcome by embedding or encapsulating the metal nanoparticles with polymer materials

(Mbhele et al., 2003). Silver nanoparticles protected by polymers, such as Polyvinyl Alcohol (PVA) (Zhou 1999, Chou 2000), polyvinylpyrrolidone (PVP) (Wang et al., 2005) and polymethylmethacrylate (PMMA) (Kong and Jang, 2008) have been reported. PVA could be considered as a good host material for metal, due to its excellent thermo-stability and chemical resistance (Kyrychenko et al., 2017). In addition, owing to its water solubility, the silver nanoparticles can be easily prepared in aqueous medium and the preparation is virtually non-toxic.

The chemical reduction method is preferred to synthesise the metal/polymer nanocomposites, because of its convenient operation and simple equipment. This preparation is simple, but great care must be exercised to make a stable and reproducible colloid. The purity of water and reagents, cleanliness of the glassware are critical parameters. Solution temperature, concentrations of the metal salt and reducing agent and reaction time also influences particle size. Controlling size and shape of metal nanoparticles remains a challenge (Mahmoud, 2015). Metallic silver colloids were first prepared more than a century ago. The most popular preparation of silver colloids is chemical reduction of silver salt by sodium citrate or sodium borohydride.

Nanoparticles with different shapes and sizes can be prepared by controlling the reaction conditions. The colour of metal nanoparticles depends on the shape and size of the nanoparticles and dielectric constant of the surrounding medium.

2.5.1.2 Silver Complexes

Silver complexes are the other form of silver solution described in the literature but are not widely used in dentistry. Researchers have found that in the treatment of infections the availability of silver ions is completely independent of the total amount of silver chelates (Dibrov et al., 2002). The silver complexes, for example, silver chelates have been described as more effective therapeutic agents than free silver ions (Batarseh, 2004).

A few amino acids with *N*- and *O*-donor ligands, which show a very wide spectrum of effective antimicrobial activities against bacteria and yeast, were used to obtain water soluble silver complexes (Kasuga et al., 2012). O-donor ligands, such as *α*-hydroxycarboxylic acids (mandelic, glycolic, malic, tartaric, etc.) also form complexes with Ag^+. It has been demonstrated that simple carboxylic acids show an unexpected ability to enhance the antimicrobial power of a wide range disinfectants and antibiotic agents (Shanbrom, 2003). The presence of sodium citrate was demonstrated to be necessary to yield potent inhibition of growth of certain pathogenic organisms (Blaszyk and Holley, 1998). Similarly, the antimicrobial antioxidant effect of citrate ions have been confirmed to be very efficient against the proliferation of various spoilage micro-organisms (Sallam, 2007).

The taurolidine-citrate solution was suggested as promising combination agent for the prevention of intravascular catheter-related infections (Shah et al., 2002). The synthesis, structure, and antimicrobial activity of silver complexes with mandelic acid were described by Cuin et al (Cuin et al., 2007). The silver mandelate compound was found to be a very successful agent against *Mycobacterium tuberculosis*. It is believed that a combination of Ag^+ ions with citrate ions ($C_6H_5O_7^{3-}$) can be very attractive for the future biomedical or pharmaceutical/therapeutic applications. Based on the literature review, it is reasonable to assume that silver citrate complexes would act synergistically as antimicrobial agents (due to the presence of Ag^+ ions) (Djokic, 2008).

As a result of AgNPs poor stability in solution and their highly technique sensitive formation techniques (Abou El-Nour et al., 2010), in addition to their expensive price (NanoXact® 2mg equals to $185) compared to 1000 times cheaper silver citrate/citric acid complexes with easier lab formation techniques as shown in section 4.8.3.

For the above reasons silver citrate complexes were chosen above AgNPs to investigate their antibacterial activity especially when they are added to GIC.

2.5.2 Silver Mechanisms of Action

The antimicrobial activity of silver either in silver complexes form or silver nanoparticles form is of significant interest because it appears to be independent of the strain of bacteria (Chaloupka et al., 2010).

Li et al (Li et al., 2008) point out four possible antibacterial mechanisms of silver ions and nanoparticles (see Figure 2.2 for a schematic summary of possible mechanisms);

1- Bacterial membrane damage through AgNPs Adhesion to bacterial surface and altering the membrane properties. The small size and extremely large surface area of nanoparticles enables them to make strong contact with the microorganism surface (Wong and Liu, 2010). Cao et al stated in their study on the antibacterial properties of silver nanoparticles embedded in titanium that the attachment of bacteria to such a surface correlates with the surface zeta potential (surface charge with time i.e. stability in colloid) of the nanoparticles. Silver modified titanium surfaces studied reduced the proliferation of both types of bacteria studied (Gram-positive *S. aureus* and Gram-negative *E. coli*) (Cao et al., 2011).

2- Interaction with cell DNA and protein; AgNPs penetrate the bacterial cell, resulting in DNA damage. In the study of Choi and Hu (Choi and Hu, 2008) the inhibition of micro-organisms was correlated with the fraction of silver nanoparticles measuring less than 5 nm in diameter, which was more toxic than any other form of silver (silver ions, AgCl colloids). The authors suggest that this may be due to easier (active) transport through the cell membrane of uncharged silver nanoparticles than of charged silver ions.

3- Interaction with cell protein; dissolution of AgNPs leads to release of silver ions (Ag^+) which can interact with sulphur-containing proteins in the bacterial cell wall, which may lead to compromised functionality. This phenomenon is often considered as the main mechanism of the antimicrobial activity of AgNPs (Lansdown, 2004, Levy, 1998, Ovington, 2004). It can be suggested that the vast knowledge of antimicrobial properties of silver ions can be applied to AgNPs case. At the same time, the problem of bacterial resistance to silver ions remains meaningful for at least some usages of silver nanoparticles.

Interaction of dissolved Ag^+ ions with cell wall and cytoplasmic proteins was also proposed by Cao et al. (Cao and Liu, 2010), who highlighted the fact that silver ions interaction with the thiol group of vital enzymes may result in their impaired function or inactivation. The exchange of silver ions between inorganic sulphur complexes and thiols was also proposed by (Adams and Kramer, 1999, Pal et al., 2007) and others. Disruption of respiration and establishment of proton motive force as an effect of interactions with thiol groups of enzymes and other proteins is also stated by Sedlak et al. (Sedlak et al., 2012). According to Lee et al. (Lee et al., 2012), silver ions inhibit enzymes acting in the phosphorus, sulphur, and nitrogen cycles of nitrifying bacteria. Silver ions can enter from the environment or originate from sustained dissolution of AgNPs taken up by bacteria.

4- Interaction with respiratory chain which leads to local proton depletion; Cao et al. (Cao et al., 2011) described that the proton electrochemical gradient in bacteria is established and maintained by respiratory processes (net transfer of protons from inside to outside of bacteria). ATP synthesis takes place

when protons enter the cell (via ATPase), so the electrochemical gradient is an essential driving force for ATP synthesis in bacteria (similar processes occur in mitochondria). If those processes are interrupted, essential energy for all energy-dependent reactions cannot be provided, which leads to microbial cell death. According to the authors, the proton-depleted regions formed around AgNPs embedded in titanium (due to micro-galvanic effect, which causes proton consumption) may disrupt the electrochemical gradient in the bacteria‘s intermembrane space and interfere with adhesion and proliferation (Cao et al., 2011). The disruption of transmembrane electrochemical gradient, the importance of which is described above, leads eventually to cell death. As stated by the authors, the hypothesis mentioned above is supported by another study, where proteomic analysis results indicated that silver nanoparticles of average diameter 9.3 nm may accumulate in the protein precursors leading to depleted intracellular ATP levels (Lok et al., 2006).

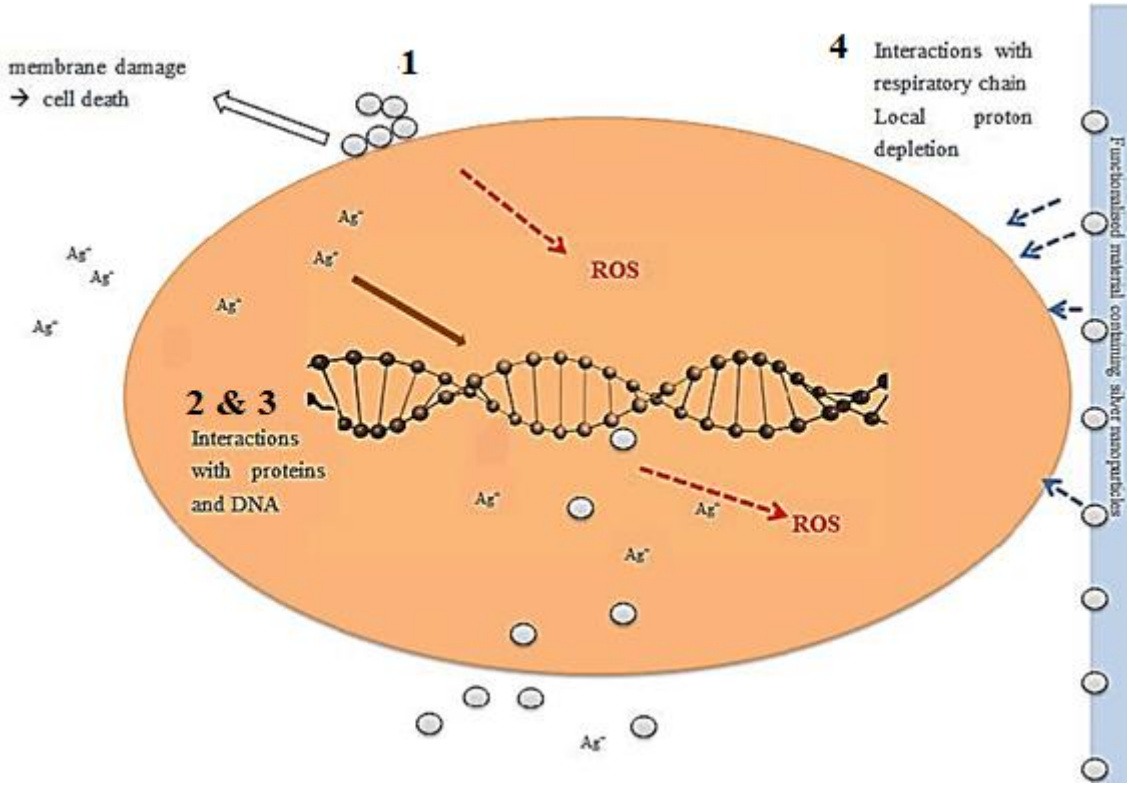


Figure 2.2 Schematic representation of the possible known mechanism(s) of antibacterial action of silver nanoparticles and released ionic silver.

The numbers 1–4 correspond to the mechanisms described previously. Grey circles indicate AgNPs and Ag^{+} implies ionic silver released from the NPs. Modified from (Reidy et al., 2013).

2.5.3 Biocompatibility of Silver

Another important aspect is the toxicity and biocompatibility of AgNPs. Considering their unique physical and chemical properties, it is likely that these Nano particles also possess unique toxicity mechanisms (Chen and Schluesener, 2008). Because of that, a better understanding of AgNPs safety is need in order to validate their clinical use (Targino et al., 2014).

Silver has a bacteriostatic and bactericidal and it is included in a range of products for which such an effect is desirable (Alexander, 2009, Holler et al., 2007), including wound dressings, refrigerators, bone cement (Holler et al., 2007, Ohbo et al., 1996). Humans also come into contact with silver via brazing or soldering, coins, tableware, jewellery, anti-smoking remedies, dental fillings, dietary supplements and ingestion of marine organisms (Holler et al., 2007, Ohbo et al., 1996). Daily human dietary silver intake has been investigated by several groups. In Italian population groups, Clemente et al. found values of less than 0.4 l g/day (Clemente et al., 1977), while values of 27 l g/day were found in a population from the United Kingdom (Hamilton and Minski, 1973). However, accidental or self/parental-inflicted poisonings with ionic or colloidal silver also occur. Such poisonings are often manifested as argyria, a blue–grey discoloration of the skin that is caused by silver deposits (Chang et al., 2006, Ohbo et al., 1996). The lowest effect level reported in animal studies is 0.5 mg of silver/kg of body-weight/day. This is based on increased plasma concentrations of cytokines in mice (Park et al., 2010a).

Johnston et al. (Johnston et al., 2010), clearly stated that the inflammatory, oxidative, genotoxic, and cytotoxic consequences are associated with silver particulate exposure, and are inherently linked. However, there is still not enough data available to conclude what the toxic effects of silver nanoparticles are, and making correlations between

findings is difficult due to the variety of different silver nanoparticles and stabilising surfaces utilised in various studies, combined with the limited characterisation of the nanoparticles dispersion characteristics and stability over the exposure time.

Because of silver's unique antibacterial activity and less bacterial resistance compared to other antimicrobials, in addition to their widely used applications in medical fields, silver compounds were selected to be added to dental restorations to enhance their antibacterial activity taking into consideration that their formulations have a concentration less than the lethal and toxic levels reported in animals.

2.6 Glass Ionomer in Dentistry

2.6.1 Glass Ionomer Cement

Glass-Ionomer cements were first developed by Wilson and Kent in the early 1970s (Wilson and Kent, 1972). They were derived from the earlier zinc polycarboxylate cements and dental silicate. Dental silicate cement results from hardening reaction between an alumino-silicate glass (powder) and an aqueous solution of phosphoric acid (Wilson, 1988). The resultant material has good properties including; an appearance similar to tooth enamel, a high compressive strength, a coefficient of thermal expansion value which is equivalent to the tooth tissues and cariostatic properties as a consequence of the slow release of fluoride ions (Wilson, 1988).

The zinc polycarbcarboxylate cements consist of a zinc oxide powder and a polyacrylic acid liquid (Smith and Ruse, 1986). Zinc ions react with the polyacid molecules upon mixing polyacrylic acid liquid with zinc oxide powder to form the set structure of the cement. The adhesion of the cement to the tooth substance is due to interaction of the polyacid with the calcium ions of the tooth structure (Smith & Ruse, 1968).

The glass ionomer cements consist of an ion-leachable glass and a poly (alkenoic) acid they react together by acid base reaction to form a cement (Walls, 1986). The resultant material has combined properties of strength, rigidity and fluoride release of the dental silicates with the biocompatibility and adhesive characteristics of the polyacrylic acids, in addition it has an acceptable aesthetics (Mclean, 1977a). Up to date a lot of modifications and changes have been carried out to both the glass ionomer powder and liquid in trials to improve their properties (Moshaverinia et al., 2012).

2.6.2 Types of Glass Ionomer Cement

This terminology is related to a material that involves a considerable acid–base reaction as part of its setting process, where the acid is a water-soluble polymer and the base is an

ion leachable glass (McLean et al., 1994). There are two types of glass ionomer cement; the conventional, prepared from a glass powder and a concentrated solution of a polyalkonic acid (Kent et al., 1973), and the resin modified glass ionomer.

2.6.2.1 Conventional Glass Ionomer Cement

The conventional glass ionomer cement is considered to be the simplest form of glass ionomer cement; it is formed by an acid-base reaction between an ion leachable glass and a poly acid (Culbertson, 2001). In another words, the chemical reaction is a neutralisation of the acid polymer groups by a chemically basic powder. The powder contains calcium alumina-silicate glass that also contains fluoride. Incorporation of fluoride is considered to be an important feature of this material because it enables it to release fluoride to theoretically prevent the occurrence of recurrent caries around restorations (Creanor et al., 1994).

2.6.2.2 Resin Modified Glass Ionomer Cement (RMGI)

Visible light cured (VLC) glass ionomer cement is another known terminology for this material. It is a hybrid material where its setting reaction involves both free-radical resin polymerisation and acid base neutralisation reactions. Consequently, resin modified glass ionomer cements (RMGI) partially set through an acid-base reaction and partially via a photochemical (visible light) or redox polymerisation process (Culbertson, 2001).

2.6.3 Composition of Glass Ionomer Cement

Not all the commercially available glass ionomer cements have the same exact composition, but all glass ionomer products share certain common elements (Wilson, 1988). The original recipe of this cement contains polyacrylic acid in liquid form that reacts with a powder that contained a calcium fluoroalumino-silicate glass (Kent et al., 1973). Since then, a lot of research was carried out to improve their properties, lots of changes and modifications to both the powder and liquid components of the original glass

ionomer cement have been made. These have also been carried out to circumvent patented technology and bring to the market products of rival manufacturers. All that resulted in a considerable differences in the composition and properties of commercial types of glass ionomer materials (Moshaverinia et al., 2012). However, three essential components are the main constituents of glass ionomer; ion leachable glass powder, water soluble acid and water (Sidhu and Nicholson, 2016).

2.6.3.1 Glass Composition

The original ion leachable calcium alumino-silicate glass including a high fluoride contents was known as G200 (Table 2.2) (Wilson, 1988).

This was the culmination of much research effort that resulted in a glass optimised for chemical reaction that yielded the most desirable physical properties.

Table 2.2 Composition of the original glass ionomer (G-200) (modified from Wilson (1988)).

Component	**Percent (%)**
SiO_2	30.1
$Al2O_3$	19.9
AlF_3	2.6
CaF_2	34.5
NaF	3.7

Fluoride is an important component of ionomer glass; during manufacturing it decreases the temperature of glass fusion, improves working properties of the cement paste and increases the strength of the set cement (Wilson, 1988).

The inclusion of silica (SiO_2), alumina ($Al2O_3$), and calcium fluoride or fluorite (CaF_2) allowed the formation of a fusion glass of these compounds. They produce a glass suitable for reaction with a polyacid with subsequent formation of a dental cement (Wilson, 1988).

The final glasses mix are formed by fusing the raw glasses (silica, alumina etc..) at high temperatures between 1100 °C and 1500 °C (the fusing temperature is depending on the materials used to make the fusion mixture) in a sillamite crucible, then pouring the resultant molten glass frit onto a metal plate, that is quenched in cold water. Once solidified the glass is then ground to give an ultimate particle size of no more than 50 µm in diameter for restorative cements and 20 µm for luting cements (Wilson, 1988).

The physical properties of the set glass ionomer may be improved by adding some non-matrix-forming inclusions into the glass (Walls, 1986). Metallic inclusions fused to glass offered potential to improve the physical properties of the set cement, for example the inclusion of crystallites of Corundum, Rutile, Aluminium Titanate and Baddeleyite into the glass were found to improve the flexural strength of the set cement (Walls, 1986). These found application in commercial products such as the Cermets when gold and silver were used. It would however be true to say that these materials have found limited clinical application in the UK (McLean, 1992).

Metal reinforced glass ionomer cements (e.g. Ketac Silver, Espe) which contain silver-tin alloy fibres or flakes being sintered to the glass particles (Mount, 1998a, Nicholson and Croll, 1997b) were developed to improve the mechanical properties of the cements and increase their wear resistance (Walls et al., 1987, Williams, 1992).

2.6.3.2 Liquid Composition

In the early recipe of glass ionomer cement, polyacrylic acid was 50 % aqueous solution (Walls, 1986). But due to instability and gelation of that solution during storage time, another poly acids were used to form glass ionomer cement (Figure 2.3), that gelation is due to a slow increase in the number of hydrogen bonds within the solution (Crisp and Wilson, 1976). The functionality and strength of these poly acids differs depending on the acid structure and the molecular weight and concentration of the acid (Lohbauer, 2009). The poly maleic acid has double the number of carboxyl groups than the polyacrylic acid and is a stronger acid (Wilson, 1988). It is more active and therefore needs less amount of reactive glasses than those used in combination with polyacrylic acid in order to form a set material (Wilson, 1988).

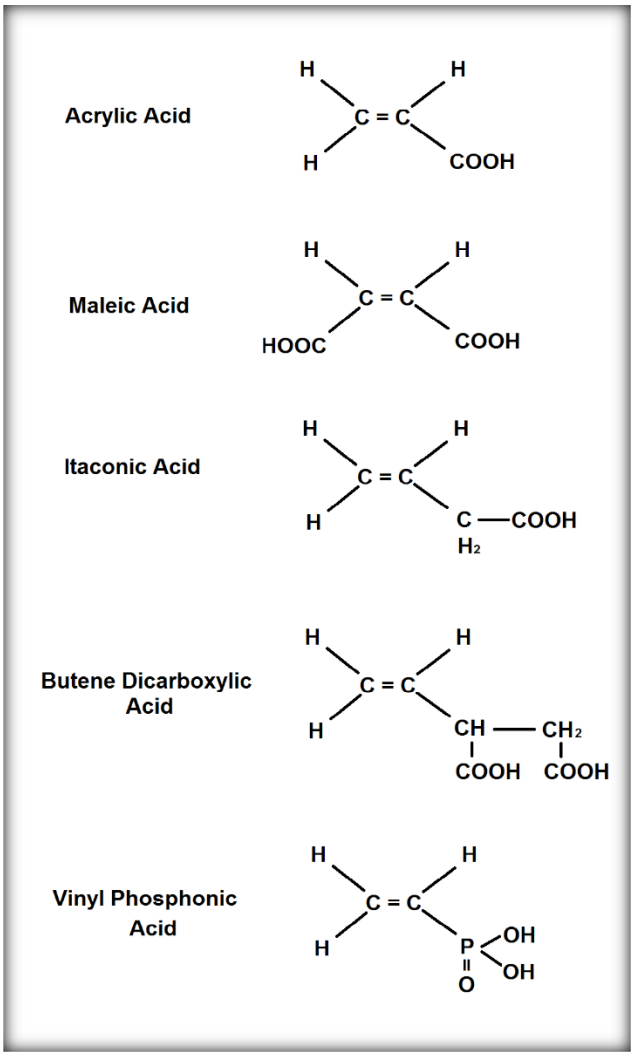


Figure 2.3 Polycarboxylic acids used to form Glass ionomer cements (Lohbauer, 2009).

Those poly acids are either in the form of a 40% to 50% aqueous solution of the poly acid or as a freeze-dried poly acid mixed with glass powder. In the latter case the setting reaction is started by mixing with either water or an aqueous solution of tartaric acid (Mclean, 1984).

The addition of tartaric acid affected the resulted glass ionomer positively, by increasing the rate of the setting reaction in addition to an increase in the compressive and tensile strength of the glass ionomer cements. Such an addition however, has no significant effect on the working time of the material (Wilson et al., 1976).

2.6.3.3 Water

The third essential constituent of the glass-ionomer cement is water. It has several roles in the formation of glass ionomer cement (Nicholson, 1998); it works as a solvent for the polymeric acid, it enhances proton release from acids, it is the medium where the setting reaction takes place, and lastly, it is a component of the cement in its set status (Nicholson, 1998).

Water incorporation with glass-ionomers leads to increased translucency of the glass-ionomer cement. The proportion of tightly-bound water increases with time for the first month or so of the cement's existence, and several possible sites have been proposed. Binding may occur partly by co-ordination to metal ions and partly by strong hydration of the polyanion molecules (Nicholson, 1998). In addition, water may react with –Si–O–Si– units at the surface of the glass particles, leading to the formation of –Si–OH groups (Czarnecka, 2015).

Water in-bounded in the set cement can be lost from the surface of a freshly placed glass-ionomer cement. Which will appear as an unsightly chalky cement because of microscopic cracks development in the drying surface. To prevent this, it is important to protect the cement by covering it with an appropriate varnish or petroleum jelly (Earl et al., 1989).

2.6.4 The Setting Reaction

The setting reaction of the glass ionomer cements is complex and differs according to the composition of the material (Culbertson, 2001). The setting reaction of glass ionomers is principally an acid-base reaction between the polyalkenoic acid (a family of complex acids which includes polyacrylic, polyitaconic, and polymaleic acid) and the glass particles (Wilson, 1988).

It involves three overlapping phases (Figure 2.4) (Crisp and Wilson, 1976);

1- Initial poly-acid attack upon the outer layer of the glass particles in which ion leaching occurs.
2- Precipitation process and salt hydrogel formation.
3- Reaction and diffusion processes that continue for several months.

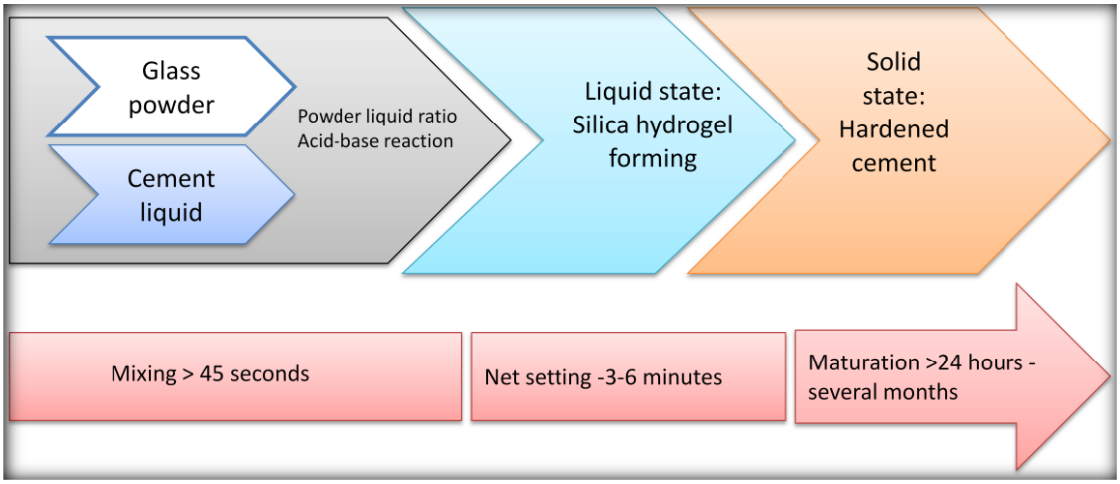


Figure 2.4 Glass ionomer setting reaction stages (Culbertson, 2001).

The setting reaction starts by wetting of the poly acid of glass particles surface layer in the presence of water, this leads to cations release (mainly calcium, aluminium, and fluoride ions from the glass) the end result is a salacious hydrogel (Culbertson, 2001).

The liberation of metallic ions is facilitated by the presence of chelating agents such as D-Tartaric acid (Prosser et al., 1982, Wilson et al., 1976).

Then the pH increases in addition to increase in the concentration of soluble ions derived from the glass. The essential change is that hydrogen ions in the liquid are replaced by metallic ions (Crisp et al., 1974).

In the early stages of the reaction the calcium salt (calcium polyacid) alone is formed (Crisp et al., 1974). This corresponds to gelation and the initial set of the material. Final hardening occurs as the aluminium salt (aluminium polyacid) is formed (Crisp et al., 1974).

The hydrogel may be considered as the materials binding matrix. Inter and intramolecular salt-bridges are formed within the poly acid by the release of the calcium and aluminium ions, which form a hard cross linked, ceramic like cement, with some molecular structures retained in the matrix (Figure 2.5) (Culbertson, 2001).

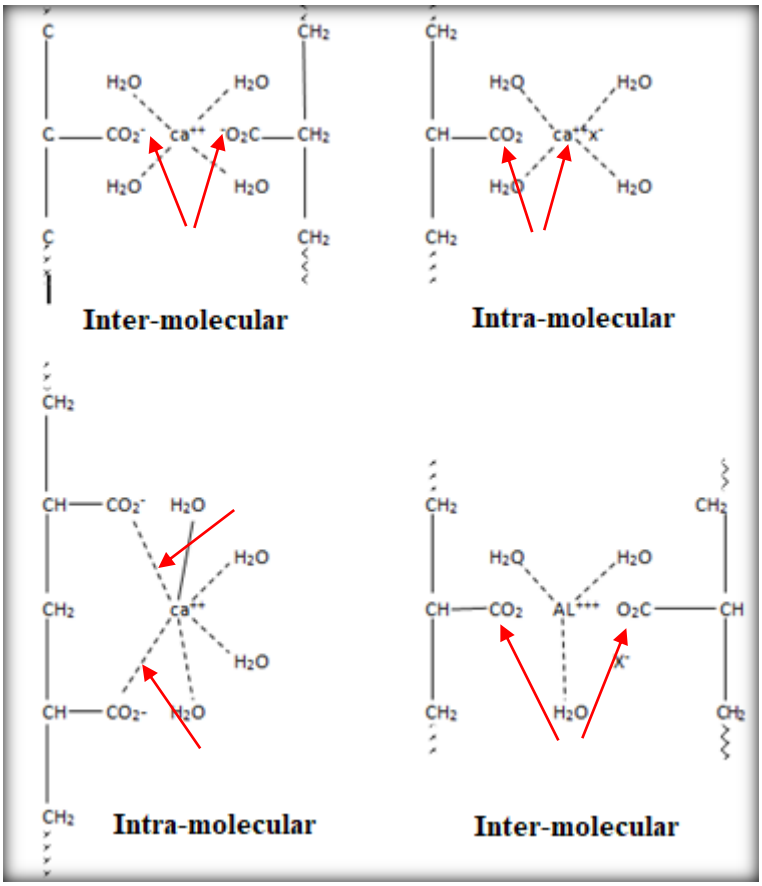


Figure 2.5 Inter-and intramolecular calcium or aluminium carboxylates (salt-bridges (red arrows)) in set glass ionomer cements. Where X represents OH^- or F^- (modified from Culbertson (2001)).

After that further hardening continues in the first 24 hours following mixing and beyond. During this time translucency develops as the chemical reactions continue, the ratio of bound and unbound water increases, and the mechanical properties (compressive strength) increase gradually until their maximum values are eventually reached (Wilson, 1988).

There are a number of factors affecting the setting reaction and the final strength of the glass ionomer cement (Wilson, 1988). These factors include; the type and composition of polymers or copolymers, the presence or absence of tartaric acid or chelating agents, the

composition of the glass powder and the powder liquid ratio used to mix the material (Smith, 1990).

The final set structure of the glass ionomer cement consists of the original glass particles surrounded by a siliceous hydrogel that is bounded by a matrix phase consisting of hydrated fluoridated calcium and aluminium poly acrylates (Smith, 1990) (Figure 2.6).

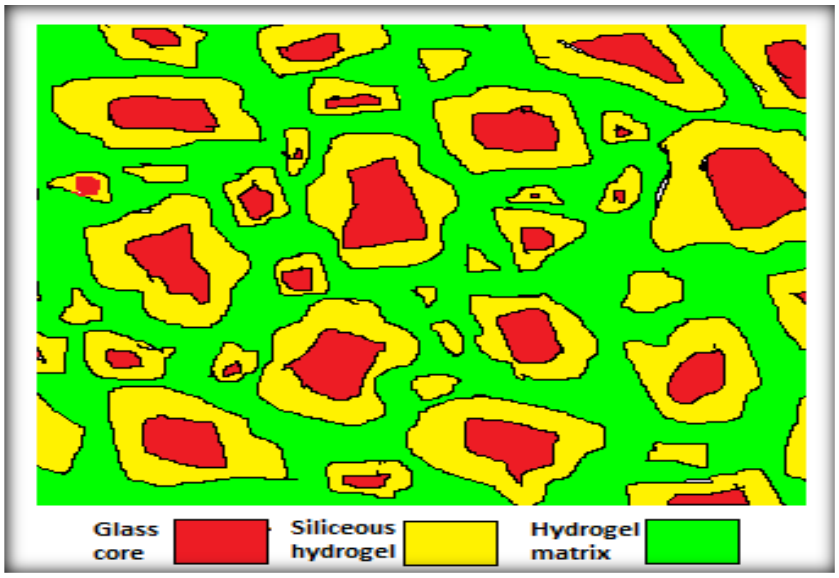


Figure 2.6 The fully set glass ionomer cement; the matrix phase and the structure of the filler (glass core attached by a siliceous hydrogel) smaller particles are completely degraded to siliceous hydrogel. (Modified from(Wilson, 1988)).

2.6.5 Properties of Glass Ionomer

Conventional glass ionomer cements pose many properties which makes them clinically attractive materials in addition of having unique properties that make them useful dental restorative materials. Those properties include, quick setting in the oral environment (3-8 minutes); it has a translucency that matches the tooth enamel especially in the case of

recent commercial materials. However, they are slightly brittle materials though they deform a little under load. Although they have a high compressive strength, but slightly weak flexural strength; compared to other dental cements they have the ability to adhere permanently to tooth structure. Finally, they also release fluoride over prolonged periods of time (Wilson, 1988).

2.6.5.1 The mechanical properties

Glass ionomer cements are considered to be slightly brittle materials but they have the ability to adhere to tooth structures (Walls, 1986). In vitro flexural or compressive strength testing of glass ionomer cements has been revealed to closely model the clinical loading situation (Lohbauer, 2009).

Multiple trials to alter the composition of the glass powder has been made; incorporation of some metallic particles has been found to have some effect on the mechanical properties of glass ionomer cement (Walls, 1986). Williams et al (1992) compared the *in vitro* mechanical properties (compressive and diametral strengths in addition to tensile strength using the four-point test) of metal reinforced and non–metal reinforced glass ionomer cements. It was found that the reinforced materials displayed significantly higher strengths than all the other materials though there was less difference in compressive strength.

Powder liquid ratio also plays a role in the resultant strength of glass ionomer cement. The concentration and molecular weight of the poly acid, and the proportion of tartaric acid, together with the amount of hydration and proportions of loose and tight water of the cement have been shown to have some effect on the mechanical properties of the glass ionomer cements (Walls, 1986).

The effect of boric and phosphoric acids addition to conventional glass polyalkenoate on compressive strength have been investigated (Prentice et al., 2006). It was found that the addition of 1% boric acid decreased the compressive strength whereas the incorporation, of up to 2% of phosphoric acid resulted in an increase in this property. This was attributed to increase cross linking of the acid chains with aluminium. Moshaverinia et al. (2008b) demonstrated that copolymers of acrylic acid and N Vinylpyrrolidone, with side chains of itaconic acid, improved the physical properties (compressive and diametral strength) by increasing the space available for ionic bond formation, with ions from the glass particles, and permitting more flexibility in the side chains. Concentration of these polymers was critical with detrimental properties if too high. The same workers also explored the synthesis and incorporation of nano-hydroxyapatite and fluorapatite particles into commercially available glass-ionomer powders. This enhanced mechanical strength (Xie, 2005).

2.6.5.2 Fluoride Release

Fluoride release and antibacterial activity are discussed in details in sections (2.1.3.5) and (2.3.1.1.1).

2.6.5.3 Adhesion

It is well known for glass ionomer cements to have the ability to adhere chemically to tooth structure as stated earlier (Wilson, 1988). But the exact mechanism of glass ionomer cement adhesion is still unclear (Walls, 1986).

Wilson and Kent (1972) suggested that the metallic ions in the set cement could make salt bridges between the poly acid and the enamel surface through negatively charged groups. It looks like the adhesion process occurs following displacement of phosphate ions from the surface of the tooth, with the place of each displaced phosphate ion being replaced with a calcium ion to retain electrolytic balance (Mount, 1998b). Van Meerbeek

described An adhesion mechanism (Van Meerbeek and P., 2006). They describe an initial micromechanical interlocking with the hydroxyl apatite coated collagen fibril network of dentine fostered by the self-etch effect of the polyacid component of the glass ionomer which resulted in substantive ionic bonds formed between the poly carboxyl groups of the glass ionomers poly acid and the calcium ions of the tooth.

Smear layer always covers the tooth surface after preparation for a restoration, treating and cleaning the surface prior to bonding enhances the adhesion between the glass ionomer cements and the cavity enamel and dentine surfaces. Those agents includes an aqueous solution of tartaric acid, polyacrylic acid that remove the smear layer and other contaminants that leave the dentinal tubules closed but the rest of the surface clean. This also changes the surface energy of the tooth structure to allow better adaptation of the cement and facilitate optimum placement of the restoration (Mount, 1998b, Wilson, 1988). Other recent laboratory work supports the use of a polyalkenoic acid conditioner to enhance bonding of resin modified glass ionomer cements to bur cut dentine as its application removes smear layer interference (Cardosoa, 2010).

2.6.6 Clinical Application in Dentistry

Glass ionomers have been used in clinical dentistry for more than 30 years since their first introduction. The original glass ionomer cements did not receive widespread acceptance until the mid-1980s, especially in the United States (Nicholson and Croll, 1997b).

The clinical development and use of the glass ionomer cement cements was first explained by (Mclean, 1977a, Mclean, 1977b). However, these materials had some advantages such as ion exchange adhesion to the enamel and dentine, ability to release and up take fluoride ions, and good thermal expansion and contraction (Nicholson and Croll, 1997b).

Previously, glass ionomer cements didn't have wide acceptance because of their low tear resistance and brittleness. Nowadays they include a wide family of materials that includes direct restoratives, luting agents, liner and bases, as well as pit and fissure sealants. All such products are available in both conventional and resin modified form (Mount, 1999). The differences consist of powder particles size, reactivity of the surface of powder particles, speed of setting and resistance to water loss and uptake. The glass ionomer cements may be classified according to their use in restorative dentistry as; 1-Type I for luting cement. 2-Type II for restoration, 3-Type III lining cement (Mount, 1999).

2.6.6.1 Luting Cement

Luting cements have fine powder particles size and are mixed at a low powder liquid ratio of around 1:1.5 (Mount, 1998). The luting glass ionomer cements are used for cementing stainless steel crowns for deciduous teeth, precision cast crowns fixed prostheses for permanent teeth, space maintainers and single orthodontic bands (Nicholson and Croll, 1997a).

2.6.6.2 Restorative Material

Different formulations of glass ionomer restorative materials are available commercially; auto-cured glass ionomer cements, resin modified glass ionomer cements and reinforced glass ionomer cements (Mount, 1998). The auto cured glass ionomer cements are ideal to restore areas which are not subjected to excessive occlusal load such as the Class V and Class III lesions. The resin modified glass ionomer cements are stronger than the auto-cured glass ionomer and can therefore be used in places where there is a moderate occlusal load. The reinforced glass ionomer cements display higher physical properties but lack translucency. As a consequence, their clinical applications are limited to use in places where strength is required more than aesthetics. They may also be used as long-term

temporary restorations where their fluoride release is used to stabilise and remineralise cariously affected dentine (Mount, 1998).

Due to the amalgam-like optical performance, metal-reinforced material glass ionomer cements (Ketec Silver) showed enormous commercial success and were consequently used as posterior materials for the primary dentition as well (Kramer et al., 1996). However, a clinical study by Kilpatrick (Kilpatrik, 1993), demonstrated that Ketac Silver was inferior to the conventional non-metal-reinforced glass ionomer cement Ketac Fil in Class II cavities. Clinical reports dealing with non-resin-modified glass ionomers in Class II restorations of primary molars show unfavourable results in general (Holst, 1996) (Randall and Wilson, 1999) which led to development of glass ionomer cements with a higher powder-to-liquid ratio, a lower water content, and smaller glass particles leading to high viscosity glass ionomer cements (non-metal-reinforced: Fuji IX, Ketac Molar; metal-reinforced: Hi Dense) which should be packable like amalgam and reveal enhanced strength characteristics (Guggenberger, 1998; Holst, 1996; Williams, 1992).

2.6.6.3 Lining and Base Cement Material

Powder to liquid ratio is the only integral difference between lining and base cement glass ionomer cement materials. The lining cement is a thin layer of cement placed underneath the metallic restoration to work as a thermal insulator and to protect the underling dentine and pulp from traumatisation due to temperature (Mount, 1998). They are also used to seal and obturate occlusal fissures that show early signs of decay (Wilson, 1988).

Chapter 3 AIMS AND OBJECTIVES

- Develop a robust *in vitro* model to determine effectiveness of different approaches which manage biofilm formation on dental materials.

- Improve the effectiveness of dental materials to reduce biofilm formation by the enhancement of their antimicrobial properties through the addition of antimicrobial additives.

- Evaluate the antibacterial activity of fluoride release from Glass Ionomer Cement and test the effect of silver additives using the developed *in vitro* model.

- Evaluate the stability and antibacterial activity of silver citrate/citric acid complexes in solution over time.

- Investigate the effect of silver additives in the form of silver citrate/citric acid complexes on the physical properties of Glass Ionomer Cement.

Chapter 4 MATERIALS AND METHODS

The materials and methods used in this project are described in general terms in this chapter, while more specific details will be in a separate part of each experiment. Generally, the first methods and materials were used to develop the *in vitro* model system while the second aimed to develop and test silver antimicrobial additives.

4.1 *In vitro* Biofilm Model

4.1.1 Bacterial Strains

4.1.1.1 Selection

The selection between a monoculture biofilm, a defined consortium biofilm (from two up to ten species) or a microcosm biofilm (using saliva or plaque sample as inoculum) was the first step in building up the biofilm.

A microcosm has been defined as a laboratory subset of the natural system from which it originates but from which it also evolves (Peters and Wimpenny, 1988, Berridge and Tan, 1993). In this regard, plaque microcosms are bacterial biofilms that evolve *in vitro* from the natural oral microflora using the natural saliva as inoculum. Although they retain the complexity of natural plaque and have biodiverse microbiota and a heterogeneous structure when seen by electron microscopy (Sissons, 1997, Sissons et al., 1991), they have a problem in reproducibility and studying the effect on specific bacterial species. So a consortium rather than microcosm was selected to overcome the limitation of microcosm models (Table 4.1).

Species were chosen to represent bacterial species commonly isolated from plaque, and species associated with dental caries, recurrent caries and periodontal diseases including perimplantitis. In addition, strains with no clear-cut association with oral diseases were included in the consortium depending on latest hypothesis that explains oral diseases

'ecological plaque hypothesis' (Hardie et al., 1977, Marsh, 1991, Marsh, 1994, Marsh and Bradshaw, 1997, Marsh et al., 1989, Newman, 1990, Kleinberg, 2002) (McKee et al., 1985). The selected species are listed in Table 4.1, all of them were from Dundee Dental School Microbiology laboratory, except *N. subflava* and *T. forsythia* they were kindly donated by Dr Graham Strafford University of Sheffield.

Table 4.1 Selected bacterial species and their source. NCTC refers to National Collection of Type Cultures, and ATCC refers to American Type Culture Collection. ---- means not available.

Species	**Source / Reference NCTC**	**Source / Reference ATCC**
S. mutans	10449	25175
S. oralis	8029	49386
E. faecalis	8213	----
A. actinomycetemcomitans	10982	29524
N. subflava	----	49275
F. nucleatum.	----	25586
T. forsythia	----	92A2
V. parvula	11810	10790
L. casei	Extracted from yoghurt	

For simplicity the consortia were divided into subgroups;

1- **Early Colonisation Model**: *S. oralis, S. mutans, N. subflava.*

2- **Caries Model**: *S. oralis, S. mutans, L. casei, F. nucleatum, N. subflava.*

3- **Peri-Implantitis Model**: *S. oralis, A. actinomycetemcomitans, V. parvula, F. nucleatum.*

4.1.1.2 Storage

4.1.1.2.1 Frozen Stocks of Bacterial Species

The viability of bacterial cultures for a specific period of time in a given storage condition is depending on the bacterial strain. Cell death during storage could happened but, it should be minimised as much as possible. Generally, the viable storage period of bacteria increases as the storage temperature decreases, but when the temperature is below freezing point, cryoprotectants are important to reduce cell damage. Bacterial cultures for regular weekly or daily use were stored on agar plates in a standard refrigerator at 4°C. If cultures were intended to be used for more than a few weeks, more long-term storage methods were considered for maximum bacterial viability (Table 4.2).

Table 4.2 Approximate time bacterial cultures remain viable in different storage conditions.

Condition	**Temp** (°C)	**Time** (approx.)
Agar plates	4	4 - 6 weeks
Standard freezer	-20	1 - 3 years
Super-cooled freezer	-80	1 - 10 years
Freeze dried	≤4	15 years+

4.1.1.2.1.1 Frozen Stock Preparation.

Frozen stocks for each species were prepared so they can be used over a long periods of time. For better bacterial recovery the greater the cell density, the better the recovery after thawing. For most bacteria, a density of 10^7 cells/mL will result in adequate recovery if all conditions are properly maintained (Simione, 1992, Simione et al., 1991).

Cryoprotectants are additives that are mixed with the bacterial suspension before freezing to lower the freezing point and protect cells during freezing. To minimise the detrimental effects of increased solute concentration and ice crystal formation. When cultures reach freezing point, water in the cells is converted to ice and solutes accumulate in the residual free water. This localised increase in salt concentration can denature biomolecules (De Paoli, 2005). Furthermore, ice crystal formation can damage cell membranes.

The most commonly used cryoprotectants are dimethylsulfoxide (DMSO) and glycerol (Hubalek, 2003).

Frozen stocks were prepared using the following protocol;

- 80% glycerol solution was first autoclaved and allowed to cool, the reason for using 80% glycerol is that it is simply much easier to handle than is 100%.
- Each single species was grown on a selected appropriate agar plate and then a single colony of each species was picked and inserted in an appropriate selected broth medium (e.g., CB, BHI etc.) then incubated for 24Hrs to reach stationary phase Figure 4.1.
- 0.5ml of that culture was added to 0.5ml of 80% sterile glycerol in the sterile screw cap micro-centrifuge tube labelled with the name of the species.
- Then the tube was closed and vortexed till it was perfectly mixed.
- The mix were then snap-frozen by immersing the tubes in liquid nitrogen for seconds and then stored at -70°C.

4.1.1.3 Growth

Generally, to achieve bacterial growth in laboratories bacteria need to multiply in a proper growth media under controlled optimal growth condition. Each bacterial species has its specific growth conditions however they all share the same growth pattern which is called as a growth curve. Growth curve is divided into four different phases; lag phase (A), log phase or exponential phase (B), stationary phase (C), and death phase (D) (Figure 4.1).

A- **Lag phase**; during this phase bacteria adapt themselves to growth conditions. Each individual bacterium is in a maturation phase where synthesis of RNA and DNA, enzymes and other molecules occurs and preparing themselves for division.

B- **Log phase** (sometimes called the logarithmic phase or the exponential phase); it is a period characterised mainly by cell doubling. That means in each specific period of time the number of bacterial cells are doubled. If growth is not limited,

doubling will continue at a constant rate so both the number of cells and the rate of population increase doubles with each consecutive time period. For this type of exponential growth, plotting the natural logarithm of cell number against time produces a straight line. The slope of this line is the specific growth rate of the organism, which is a measure of the number of divisions per cell per unit time. The actual rate of this growth (i.e. the slope of the line in the Figure 4.1) depends upon the growth conditions, which affect the frequency of cell division events and the probability of both daughter cells surviving. Exponential growth cannot continue indefinitely, however, because the medium is soon depleted of nutrients and enriched with wastes.

C- **The stationary phase**; because of growth limitation factors like nutrient depletion and/or production of inhibitory components like organic acids, bacterial cells growth rate decreases and a stationary phase results from a situation in which growth rate and death rate are equal. The result is a smooth horizontal linear part of the curve during the stationary phase.

D- **Death phase**; literally bacteria die and the curve declines.

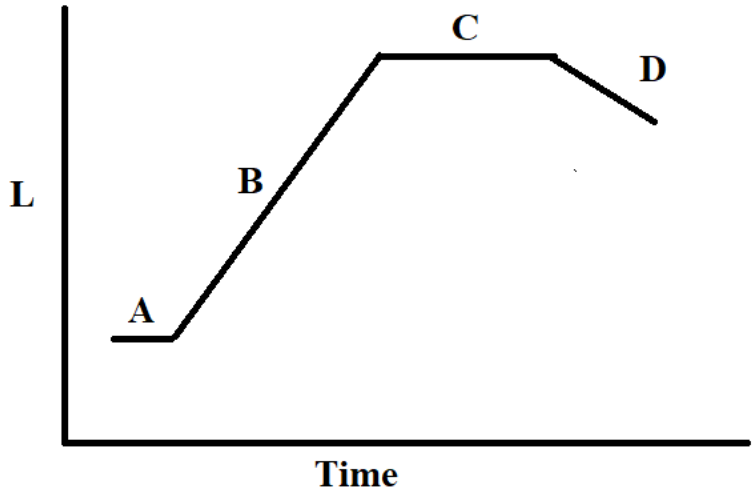


Figure 4.1 Bacterial growth curve, Growth is shown as L = log (numbers) where numbers are the number of colony forming units per ml CFUs/ml, versus Time.

4.1.1.3.1 Growth Media

Bacterial growth media are usually in a form of broth (liquid) where the bacteria grow in a solution, or agar media (gel) where the bacteria grow on a surface of gel made from agar and broth media. There are a wide range of commercially available broth and agar media however one type of media could be provided by the manufactures in both formulations i.e. agar base or broth.

4.1.1.3.1.1 Broth Media

A range of standard growth media were used in this project to determine the best for each species; Columbia Broth (CB), Brain Heart infusion (BHI), Luria Broth (LB) and Tryptic Soy Broth (TSB). But Columbia Broth (CB); Catalogue number 294420 (Difco, BD Company, de Pont De Claix, France) was mainly used because of its supportive capability to the growth of most of the consortium species. It is provided from the manufacturer as a powder, a litre of it after preparation contains the following;

1- **CB Components**: in 1L formulation

- Enzymatic Digest of Casein 5 g.
- Enzymatic Digest of Animal Tissue 5 g.
- Yeast Enriched Peptone 10 g.
- Enzymatic Digest of Heart Muscle 3 g.
- Sodium Chloride 5 g.
- Dextrose 2.5 g L.
- Cysteine 0.1 g.
- Magnesium Sulfate 0.1 g.
- Ferrous Sulfate 0.02 g.
- Tris (hydroxymethyl) aminomethane 0.83 g.
- Tris (hydroxymethyl) aminomethane-HCl 2.86 g.

- Sodium Carbonate 0.6 g.
- Final pH 7.3 ± 0.2 at 25°C.

2- CB Preparation

It was prepared according to the manufacturer's instruction by mixing powder and distilled water in appropriate proportions in a sterile bottle. After that the mixture was autoclaved and then it was stored it in 4°C fridge until use.

4.1.1.3.1.2 Agar base media

A range of agar base media were used to determine the optimum media for growth and differentiations between species depending on colony morphology; Sheep blood agar plates, CB plates, Mannitol Salt agar plates and LB plates. CB agar plates and Mannitol Salt agar plates were mainly used.

1- Mannitol Salt Agar Properties and Components.

Mannitol salt agar or MSA is a commonly used selective and differential growth medium in microbiology. It encourages the growth of a group of certain bacteria while inhibiting the growth of others. This medium is important in medical laboratories as it can distinguish pathogenic microbes in a short period of time. It contains a high concentration (about 7.5%-10%) of salt (NaCl), making it selective for Gram-positive bacteria especially streptococci (*S. mutans*, *S. oralis*) since this level of salt is inhibitory to most other bacteria. It is also a differential medium for mannitol-fermenting staphylococci, containing carbohydrate mannitol and the indicator phenol red, a pH indicator for detecting acid produced by mannitol-fermenting species. *S. mutans* produces yellow colonies with yellow zones, whereas *S. oralis* produce small pink or red colonies with no color change to the medium. If an organism can ferment mannitol, an acidic byproduct is formed that causes the phenol red in the agar to turn yellow. It was used for the

selective isolation of *S. mutans* in this project (Figure 4.2). It is provided by the manufacturer as a powder, MSA agar plates out of this powder were prepared according to the following protocol.

Figure 4.2 MSA agar plate showing the selective growth of *S. oralis, S. mutans* rather than *N. subflava,* in addition the differentiation between *S. Mutans* and *S. oralis* depending on colony morphology i.e. *S. mutans* colonies gives a yellow background because of mannitol fermentation but *S. oralis* not.

2- MSA Plates Preparation

- According to manufacturer's instructions a powder and distilled water mixed in a specific ratio in a sterile bottle and then autoclaved.
- The autoclaved solution was left on the bench to reach a hand hot point then it was poured into a sterile petri dishes spread over a fume cabinet's working top to keep it sterile while cooling down.

- When MSA agar set in petri dishes, dishes were covered with their lids then stored in the fridge 4 ºC.

4.1.1.3.2 Growing Protocols of bacteria

4.1.1.3.2.1 Streak Out from Frozen

The aim was to isolate a single bacterial colony of a specific species to make a broth culture for that specific species.

The following protocol was used:

- The tube corresponding to the intended specific species was taken out of the freezer and placed on the bench.
- Using a sterile plastic disposable loop, a portion from the top of the frozen glycerol stock was scraped off and streaked onto a plate.
- The tube was closed and quickly returned to the freezer. The stock must not be allowed to thaw since cyclic thawing and freezing will kill the bacteria.
- The portion was streaked in a specific pattern to insure spreading of the bacteria and to obtain single isolated colony (Figure 4.3).

  To streak the bacteria out from frozen there is no need to change the sterile loop between each side of spreading because the bacterial viability is compromised by low temperature i.e. -70°C.
- The plate was then incubated under appropriate conditions, suitable for the selected species to achieve growth on the agar plate.

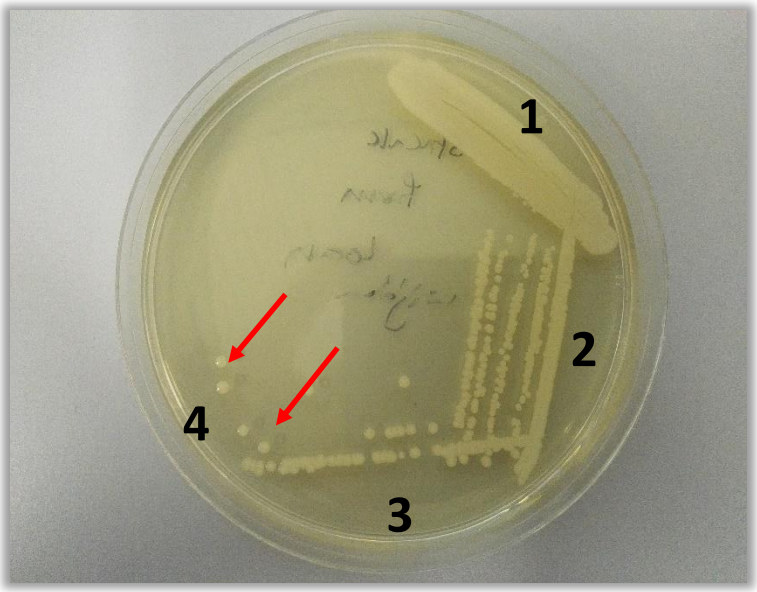


Figure 4.3 An illustration for the pattern of streaking out from frozen stocks starting from point 1 till point 4 to result in discrete colonies (red arrows) on CB agar plate.

4.1.1.3.2.2 Broth Culture

In these cultures, the desired bacteria are suspended in a liquid nutrient medium, such as CB, in an upright flask. This allows the growth of large amounts of bacteria for a variety of downstream applications. Cultures were prepared as the following protocol.

- A desired single colony was picked from agar plate using a sterile loop was then inserted and shaken in a flask of sterile liquid media.
- The flask was then left in a shaking bath with a specific shaking speed and specific temperature depending on the test conditions.
- The growth was monitored using a spectrophotometer (Eppendorf, Hamburg, Germany).
- The turbidity of the culture was assessed by a reading known as Optical Density at 600nm wavelength (OD_{600}).

4.1.2 Artificial Saliva

The aim was to find a growth medium that supported the growth of all consortia microbes especially the fastidious microbes (*V. parvula*, *F. nucleatum*, *T. forsythia*).

For *in vitro* plaque studies, various chemically undefined, simulated oral fluids have been formulated, containing mucin, yeast extract and/or peptones as major components (Russell and Coulter, 1975; Shah et al., 1976; Glenister et al., 1988; Sissons et al., 1991, 1995a). One of these, BMM (Basal Medium Mucin); a complex mucin-containing medium (Glenister et al., 1988), has been widely used for *in vitro* studies of oral bacteria. Although BMM is a standard culture medium for oral microbial studies, it contains large quantities of various unknown peptides from the manufacture of yeast extract and peptone mixtures, giving inconsistent and usually unknown concentrations of specific peptides, vitamins and ions between batches.

It is essential to use a chemically defined medium in which known changes can be made to individual components, for example, to study factors regulating plaque growth and mineralisation. A chemically defined, simulated oral fluid (defined medium mucin, DMM) was used in the project as an artificial saliva and growth medium, with DMM it is possible to manipulate macromolecular, vitamin and ion components of the medium to determine their effect on plaque microcosm growth, composition and properties, and pathogenicity. It contains commercially available components that simulate relevant constituents of saliva.

4.1.2.1 DMM Composition

The composition of DMM is based on the shellis artificial-saliva formulation ((Shellis, 1978)and some subsequent saliva analyses (Korayem et al., 1990, Syrjanen et al., 1990), modified to facilitate large scale biofilm culture. The components of DMM and the Shellis artificial saliva are listed in (Tables 4.3-4.5). Concentrations were approximated to

convenient values within the natural range of those in saliva. Generally, DMM contains the following:

- Ions.
- Mucin, commercially available, partially purified pig gastric mucin at 0.25%, a concentration similar to that in natural saliva (Shellis, 1978).
- Basal mixture of amino acids (n = 21) based on the concentration of free amino acids measured in saliva supernatant (Korayem et al., 1990; Syrja¨nen et al., 1990).
- Vitamins and other growth factors (n = 17).
- (protein/peptide) equivalent amino acids to model the salivary proteins.
- Haemin is included to provide iron; together with menadione which promotes the growth of black-pigmented *Porphyromonas* and *Prevotella* spp. Their amount is unchanged from those of BMM and other standard media for anaerobes (Holdeman and Anaerobe, 1977).

Several vitamins are at higher concentrations than reported for saliva (Table 4.5), mainly to reflect the requirements of Treponemes (Wyss, 1992) and to allow for some degradation during the experimental period of several weeks. The bacterial growth factors, *p*-aminobenzoic acid and inositol, were also included. Both are present in peptones and standard vitamin microbial culture supplements, including a semi-defined medium for streptococci and actinomyces (Bowden, 1999). Inositol is also present in the chemically defined medium of (Wyss, 1992). But no reports have been found of their presence in saliva and in preliminary measurements but (Wong and Sissons, 2001) found approximately 4 μmol/l inositol.

The inclusion of natural human or animal salivary proteins is impractical for large-scale plaque modelling. An amino acid mixture was used to replace the (protein/peptide)

component of BMM in addition to the basal salivary amino acids. Currently its composition is based on the known amino acid sequence for casein (Lehninger, 1970) at 5 g/l intact protein.

Table 4.3 Comparison of components in DMM and the Shellis (1978) artificial saliva. (Wong and Sissons, 2001).

Component	**DMM**	**Shellis artificial saliva**
Salts (mmol/l)		
$CaCl_2$	1.0	1.4
$MgCl_2$	0.2	0.2
KH_2PO_4	3.5	2.6
K_2HPO_4	1.5	
Na_2HPO_4		2.6
NaCl	10.0	
KCl	15.0	15.6
NH_4Cl	2.0	4.4
KSCN		2.3
$NaHCO_3$		6.4
Urea (mmol/l)	1.0	2.9
Mucin (g/l)	2.5	2.5
Albumin (μmol/l)		0.4
α-Amylase (Somogyi units)		3×10^5
Amino acids≡5 g/l casein[a] (mmol/l)	43.12	
Basal salivary amino acids[b], *n*=21 (μmol/l)	770	365
Vitamins/growth factors[c], *n*=17 (μmol/l)	243	219

a Based on amino acid composition of casein.

b See 4.4, based on revised literature values.

c See Table 4.5.

Table 4.4 Comparison of concentrations of basal salivary amino acids in DMM and the Shellis (1978) artificial saliva. (Wong and Sissons, 2001)

Amino acid (µmol/l)	DMM	Shellis artificial saliva
Alanine	50	37.1
Arginine	50	10.9
Asparagine	25	
Aspartic acid	25	12.0
Cysteine	50	
Glutamic acid	25	26.5
Glutamine	25	
Glycine	100	118.5
Histidine	10	6.4
Isoleucine	25	22.1
Leucine	25	22.1
Lysine	50	18.5
Methionine	10	0.2
Phenylalanine	25	17.6
Proline	100	1.7
Serine	25	20.0
Taurine	75	
Threonine	25	24.3
Tryptophan	10	
Tyrosine	15	11.6
Valine	25	15.4
Total (µmol/l)	770.0	364.9

Table 4.5 Comparison of concentrations of vitamins and growth factors in DMM, BMM and the Shellis (1978) artificial saliva. (Wong and Sissons, 2001).

Vitamins (µmol/l)	**DMM**	**Shellis artificial saliva**	**BMM**
Choline chloride	100	107	14
Citrate	50	44	
Pantothenic acid	1 [c, d]	0.34	5.8
Thiamine	1[c]	0.02	7.0
Riboflavin	0.3	0.13	1.6
Biotin	0.1[c]	0.0033	0.07
Cyanocobalamin	0.05[c]	0.0022	0.0005
Folic acid	0.025[e]	0.00023	0.02
Ascorbic acid	5[b]		
Niacin	5 [c, d]	0.24	25
Pyridoxine	4	3.55	1.3
Creatinine	1	0.88	
Growth factors (µmol/l)			
Uric acid	50	62	
Haemin	10[a]		7.7
Inositol	10		43
p-Aminobenzoic acid	1[d]		28
Menadione	5[a]	0.09	5.8

a Haemin and menadione, required to encourage *Porphyromonas*/*Prevotella* spp., are present at 7.7 and 5.8 mmol:l in BMM and kept at similar concentrations for DMM.

b (Makila and Kirveskari, 1969).

c These vitamins, ranging from 20 to 50-fold greater than the concentrations reported for saliva, are present at similar or higher concentrations in a defined medium for growing Treponemes (Wyss, 1992).

d Similar to concentrations in GCHI enrichment (Remel, GCHI Enrichment, Technical Information, TI No. 21070).

e Folic acid and folinic acid concentration is ten times greater than in the Wyss (1992) medium.

4.1.2.2 DMM Preparation

1. Individual stock solutions of urea and salts in concentrations listed in (Table 4.6) were prepared.

Table 4.6 Concentration of salts and urea in mol/l.

Salt	Concentration mol/l	Salt	Concentration mol/l
Urea	0.5	K_2HPO_4 $3H_2O$	0.5
$CaCl_2 \cdot 2H_2O$	1.0	NaCl	1.0
$MgCl_2$ $6H_2O$	0.2	KCl	3.0
KH_2PO_4	0.5	NH_4Cl	0.2

2. Individual stock solutions of basal salivary amino acids listed in Table 4.7 were prepared at concentration of at 100 mmol/l.

Table 4.7 Basal salivary amino acids.

Alanine	Glutamine	Leucine	Proline	Valine
Arginine	Glycine	Lysine	Serine	
Asparagine	Histidine	Methionine	Taurine	
Cysteine	Isoleucine	Phenylalanine	Threonine	

3. Individual stock solutions of the following basal salivary amino acids were prepared:

Aspartic acid: 20 mmol/l

Glutamic acid: 50 mmol/l

Tryptophan: 20 mg/l

Tyrosine: 10 mg/l.

4. A combined solution of the basal salivary amino acids was prepared by mixing together the following volumes in ml in Table 4.8 of the individual stock solutions.

Table 4.8 Basal salivary amino acids required volumes to make 107 ml final combined volume**.**

Amino Acid stock	**Volume ml**	**Amino Acid stock**	**Volume ml**	**Amino Acid stock**	**Volume ml**
Alanine	5	Histidine	1	Taurine	7
Arginine	5	Isoleucine	2.5	Threonine	2.5
Asparagine	2.5	Leucine	2.5	Tryptophan	5
Aspartic Acid	12	Lysine	5	Tyrosine	15
Cysteine	5	Methionine	1	Valine	2.5
Glutamic Acid	5	Phenylalanine	2.5		
Glutamine	2.5	Proline	10		
Glycine	10	Serine	2.5		

5. Individual stock solutions of the following vitamins/growth factors were prepared in mmol/l:

Choline chloride: 100

Sodium citrate: 50

Haemin: 10

Inositol: 10

6. Combined as a 1000-fold stock solution of the following was prepared;

Biotin

Creatinine

Cyanocobalamin

Folic acid

Menadione

Niacin,

p-aminobenzoic acid

Pantothenic acid

Pyridoxine

Riboflavin

Thiamine

7. Ascorbic acid was prepared fresh as a 5mmol/l, 1000-fold stock solution.

8. An amino acid mixture listed in Table 4.9 was used to replace the 'protein/peptide' component of BMM in addition to the basal salivary amino acids. Currently its composition is based on the known amino acid sequence for casein (Lehninger, 1970) at 5 g/l intact protein.

Table 4.9 Amino acid mixture and concentration of each amino acid in final volume 1L.

Amino Acid stock	concentration mmol/l	Amino Acid stock	concentration mmol/l	Amino Acid stock	concentration mmol/l
Alanine	1.59	Histidine	1	Threonine	1.08
Arginine	1.3	Isoleucine	2.5	Tryptophan	0.43
Asparagine	1.73	Leucine	2.5	Tyrosine	2.17
Aspartic Acid	1.52	Lysine	5	Valine	2.38
Cysteine	0.05	Methionine	1		
Glutamic Acid	5.41	Phenylalanine	1.73		
Glutamine	3.03	Proline	3.68		
Glycine	10	Serine	3.46		

9. For each litre of DMM, 2.5 g of mucin was dissolved in 100-ml distilled water, heated to 80°C for 1 h, cooled and stored at 4°C overnight before filtering through no. 4 and then no. 1 filter papers (Whatman Ltd, Maid-stone, England).

10. The (protein/peptide) equivalent amino acids (except asparagine, cysteine, glutamine and tryptophan) were weighed and dissolved together in 250-ml distilled water with heating, then added to the mucin solution, together with appropriate portions of the stock ion solutions (except $CaCl_2$) to give the final concentration needed in the medium, and finally 1 ml each of choline chloride, sodium citrate, haemin, and inositol were added.

11. Uric acid was weighed out and added directly.

12. The volume was made to 965 ml with distilled water, the pH of the solution adjusted to 6.8 with 5 mol/l NaOH, and the solution autoclaved.

13. The remaining ‘protein/peptide equivalent’ amino acids (asparagine, cysteine, glutamine and tryptophan) were weighed and dissolved together in 20-ml distilled water and thoroughly mixed with 10.7 ml of basal salivary amino acids, 1 ml of combined 1000-fold vitamin/growth factor, 2 ml of urea, 1 ml each of CaCl2 and ascorbic acid stock solutions. The mixture was adjusted to pH 6.8 and then filter-sterilised (0.2 µm) and added to the already autoclaved medium from step 12 at room temperature.

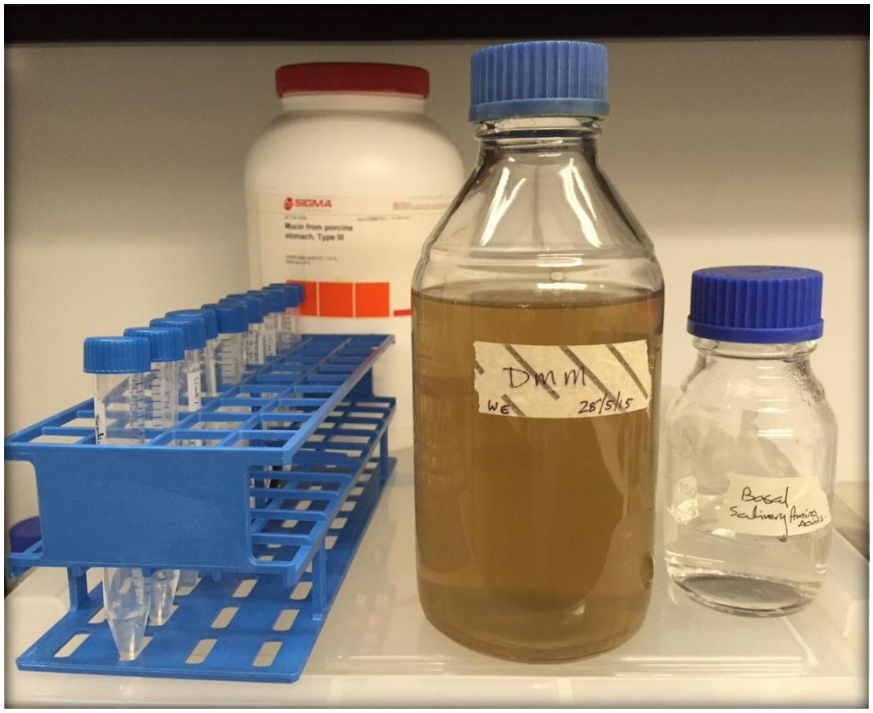


Figure 4.4 1 litre of DMM stored in -4ºC.

4.1.3 GIC Specimen's Preparation

4.1.3.1 Specimens for *in vitro* Model

Specimens were fabricated using Polytetrafluoroethylene (PTFE) moulds which were prepared at the Dental School workshop at University of Dundee, with an inner diameter of 8 mm, and a thickness of 1 mm (Figure 4.5). PTFE moulds, the holding metal plate and rings were washed thoroughly with water and detergent, and then sterilised using an autoclave before use.

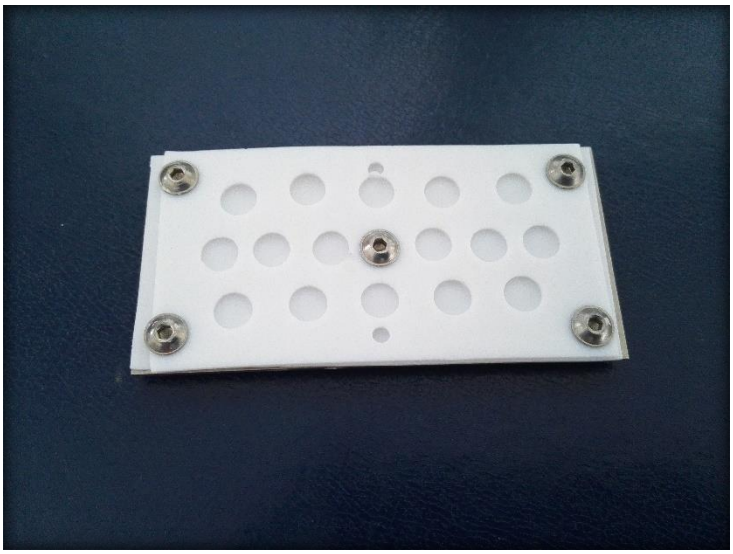


Figure 4.5 PTFE molds.

Preparation of disks was carried out under sterile condition using sterilised instruments. Sterile gloves were used in addition, scoops, droppers and plastic spatulas provided by the manufacturers were rinsed thoroughly in 100% ethanol. All baseline specimens of commercial type of glass ionomer cements (ChemFil Superior®) were mixed according to manufacturers' instructions and condensed using plastic instruments, into the PTFE mould which was placed on a PTFE sheet and then the PTFE mould was covered with another PTFE sheet before they were all screwed in between two metal plates. After initial

setting (5 minutes) the disks were removed from the mould by gentle hand pressure, after unscrewing the metal plates. The specimens then were stored in sterile distilled water in an incubator at 37ºC for 24 hours before use, in order to allow the glass ionomer to be fully set.

4.1.3.2 Specimens for Physical Testing

Specimen's preparation was done following the standard protocols from previously published protocols for each specific test (Salem et al., 2016).

For the specimens tested for compressive strength a split stainless steel mould, giving specimens sizes of 6 mm long and 4 mm diameter was used (Figure 4.6).

Mould was lubricated, to facilitate specimen release using Vaseline.

4.1.3.2.1 Fabrication of Specimens for Compressive Strength Testing

Prior to mixing the cements under test, the compressive strength mould was placed upon a flat glass slab covered by a clear cellulose matrix strip (Hawe-Neos DentalCH-6934 Bioggio Switzerland). In all other cases the mould designs themselves contained a flat base against which the base of the specimen was formed.

Thereafter the mixed cement was applied into the well of the moulds using a plastic spatula, with packing action, to slight excess. A cellulose matrix strip was then applied to the exposed surface and pressure applied to the material through a flat glass slab on which was placed a 5 Kg weight for 5 minutes. Once this time had elapsed, the specimen was removed from the mould. If upon visual inspection no defects were found the specimen was accepted for storage and testing. They were stored for one week in distilled water at 37 C° prior to testing (Salem et al., 2016).

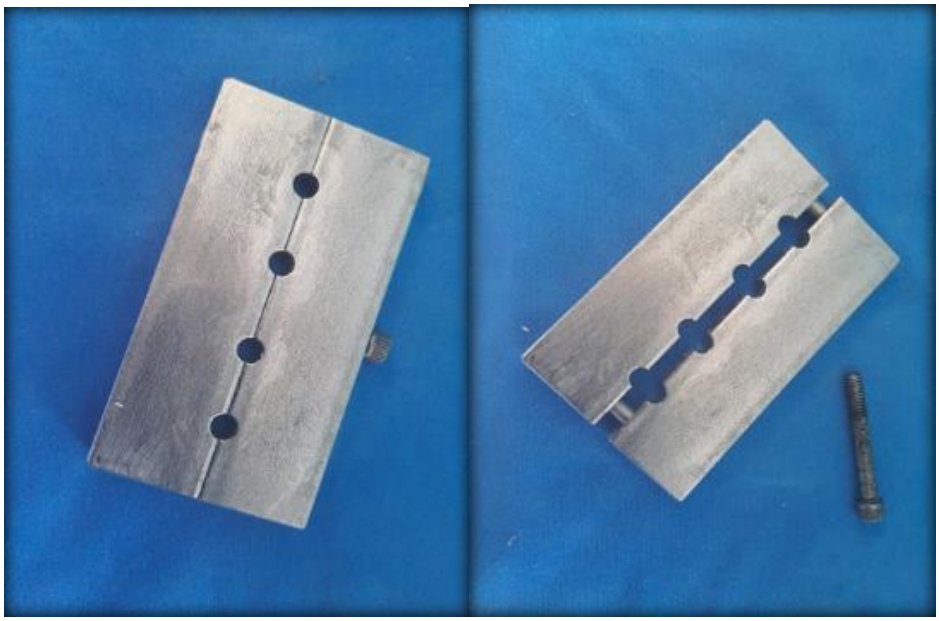


Figure 4.6 The Split stainless steel mould used to fabricate compressive strength specimens.

4.1.3.2.2 Fabrication of Specimens for Hardness Testing

Hardness testing specimens were fabricated using a circular silicone rubber moulds giving specimen sizes of 2 mm thick X 12 mm diameter (Figure 4.7) to produce 15 disks of glass ionomer cements in total comprising unmodified ChemFil Superior and modified ChemFil Superior (silver complexes). For each state 5 specimens were made and following a period of 5 minutes within the mould the specimens were ejected and stored for one week in distilled water at 37 C° prior testing (Salem et al., 2016).

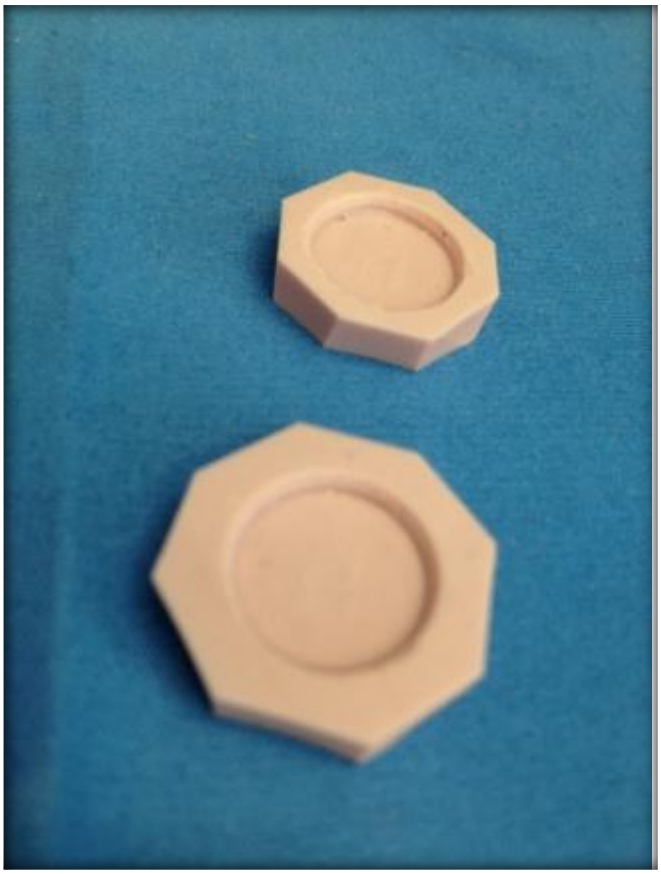


Figure 4.7 The silicone rubber moulds used to fabricate surface hardness specimens.

4.1.3.2.3 Fabrication of Specimens for Shear Bond Strength Testing

Forty extracted human molar teeth were kindly donated by Dr. Andrew Forgie (Former senior lecturer/honorary consultant in restorative dentistry, Dundee Dental Hospital and School) which were collected by him during 1994-1999. No ethical approval was required for extracted human teeth usage in this study because teeth were collected prior to 2006 as indicated by East of Scotland Ethics Service Centre (EoSESC). Teeth were stored in thymolised saline and then just prior to use they were disinfected according to Dundee Dental Hospital and School protocol. After that specimens were prepared according to the previously published protocol (Salem et al., 2016); teeth roots were removed and pulps were extirpated, then they were sectioned longitudinally and mounted in circular

epoxy resin blocks ((Bonda Clear Casting Resin, Bondaglass Vost Ltd, Kent, UK)) with their buccal/palatal surfaces upper most. The exposed surfaces were rendered flat flush with the surrounding mounting epoxy resin using a PM5 precision lapping and polishing machine (Longitech, Glasgow, Scotland) and a slurry of calcined Aluminium Oxide powder with a particle size of 9 μm (Longitech, Glasgow, Scotland), for subsequent cement application and testing. The specimen ends are shown in (Figure 4.8).

Prior to cement application all prepared samples of teeth were stored in an incubator, at 37°C, in distilled water for one month prior to cement application. This was to ensure uniform inter specimen hydration. Thereafter the specimens were removed from storage and a circular washer (3 mm diameter x 1.5 mm deep) was placed upon the exposed dentine surface. Through this the mixed ChemFil Superior was added with a flat plastic instrument, and once clinically set the washer was removed. After all specimens were prepared they were stored in distilled water at 37°C for one week prior to testing.

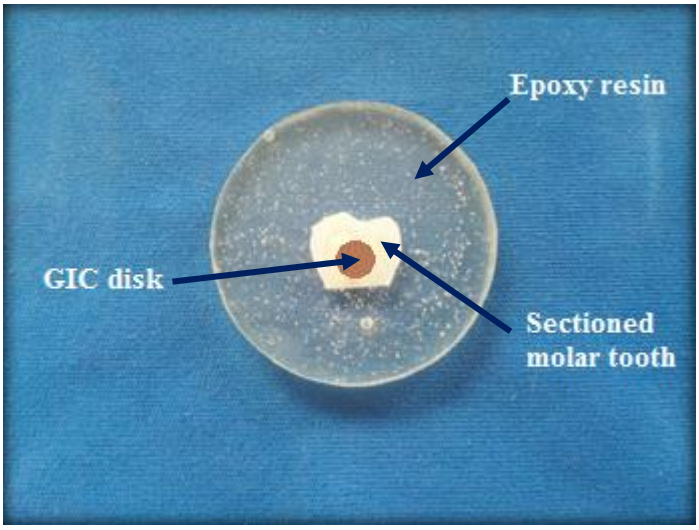


Figure 4.8 Epoxy resin disk including sectioned molar tooth with GIC disk bonded to the exposed dentine.

4.2 Viable Cell Counting (Colony Forming Units CFUs)

CFUs is a unit represent the estimated number of viable bacterial cells in a given sample. Viability means the ability of bacteria to multiply via binary fission under controlled conditions. Counting with colony-forming units requires culturing bacteria on agar plates and counts only viable cells, in contrast with microscopic examination which counts all cells, living or dead. The visual appearance of a colony in a cell culture requires significant growth, and when counting colonies, it is uncertain if the colony arose from one cell or a group of cells.

4.2.1 The Principle

The purpose of plate counting is to estimate the number of cells present based on their ability to give rise to colonies under specific conditions of nutrient medium, temperature and time. Theoretically, one viable cell can give rise to a colony through replication. However, solitary cells are the exception in nature, and most likely the progenitor of the colony was a mass of cells deposited together. In addition, many bacteria grow in chains (e.g. Streptococcus) or clumps (e.g. Staphylococcus). By counting of CFUs assumes that every colony is separate and founded by a single viable microbial cell (Breed and Dotterrer, 1916).

To determine the CFUs of a bacterial culture a dilution series of that culture must be done. A serial dilution is a series of sequential dilutions used to reduce a dense culture of cells to a more usable concentration. Each dilution will reduce the concentration of bacteria by a specific amount, in this case it’s a 10 times dilution, which reduces the density by 10000 each time.

4.2.2 **Protocol** (Illustrated in Figure 4.9)

- 900µl of sterile Phosphate Buffer Saline (PBS) were pipetted in each 1.5ml Eppendorf tube of the dilution series, the number of Eppendorf's depends on

the OD_{600} of the broth culture being tested. The tubes labelled sequentially 10^{-1}, 10^{-2}, 10^{-3} etc.

- 100µl from selected broth culture with known OD_{600} were mixed with the first Eppendorf 10^{1-} to make a final volume of 1ml then the mixture was vortexed for 10 seconds on the maximum speed.
- 100µl from that mixture were taken and mixed with the second Eppendorf of the series 10^{-2}, in the same manner of the first one and so on with the third till the last tube of the dilution series.
- 100µl from the last three dilutions were plated on selected agar plates either CB or MSA depending on the intended purpose.
- The plates were incubated under correct growth conditions for the selected species then the colonies were counted and CFU was calculated using this formula;

  CFU /ml = (number of colonies x dilution factor) / volume of culture plate.

  For example, suppose the plate of the 10^{-6} dilution yielded a count of 130 colonies. Then, the number of bacteria in 1 ml of the original sample can be calculated as follows:

  Bacteria/ml = (130) x (10^{6}) = 1.3×10^{8} or 130,000,000.

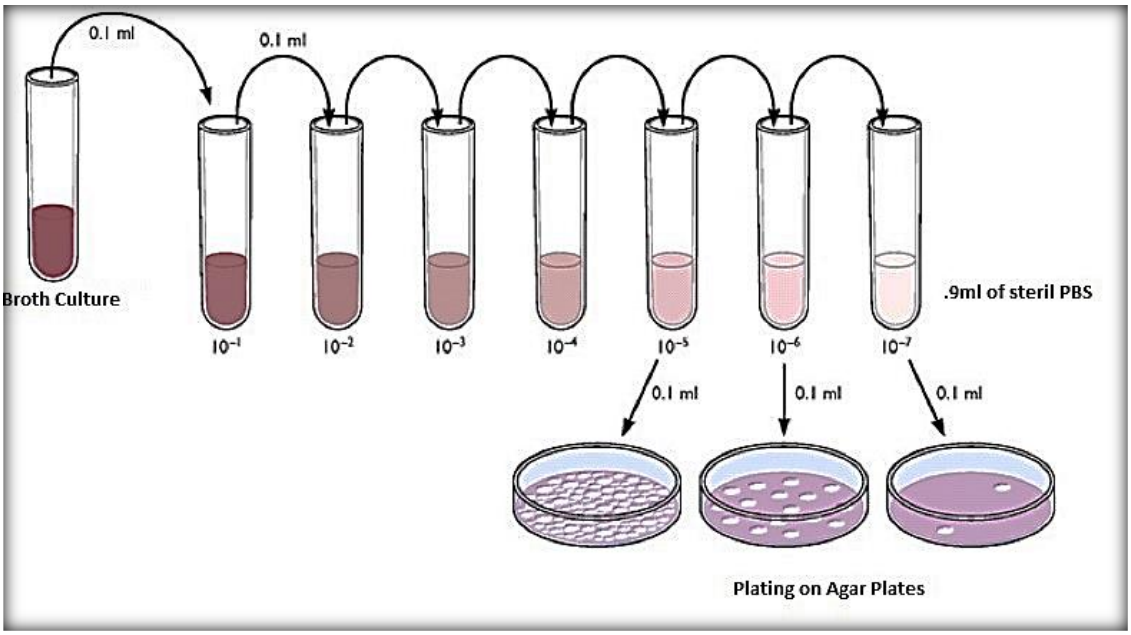


Figure 4.9 An illustration for the dilution series of a broth culture and plating out the last three dilutions (modified from Dulbecco, R., & Vogt, M. (1953)).

4.3 MTT Metabolic Activity Assay

4.3.1 The Principle

The MTT assay is a colorimetric assay of cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble form called formazan, in its soluble state it has a purple colour (Berridge and Tan, 1993) (Figure 4.10).

MTT assays are usually done in the dark since the MTT reagent is sensitive to light.

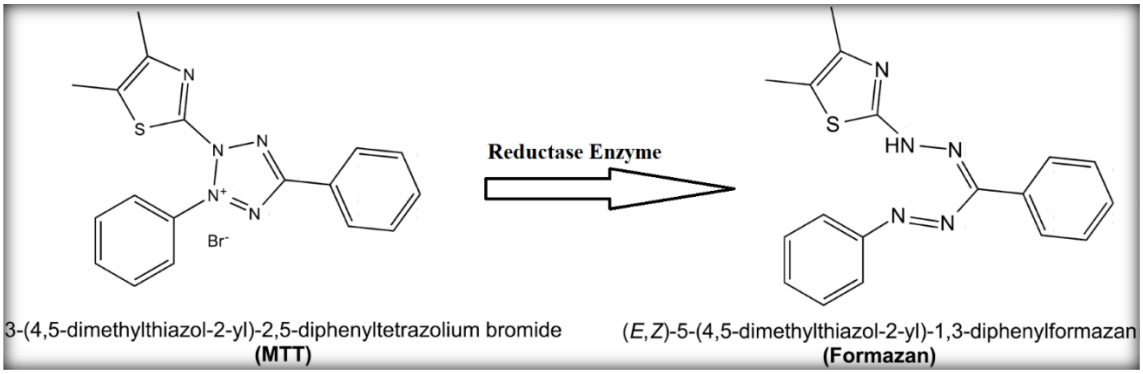


Figure 4.10 MTT and related tetrazolium salts reduction into Formazan by bacterial metabolic enzymes. Modified from (Berridge and Tan, 1993).

A solubilisation solution (usually either dimethyl sulfoxide (DMSO), an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The degree of light absorption depends on the solvent (Berridge and Tan, 1993).

The mechanism of reduction is dependent on NAD(P)H-dependent oxidoreductase enzymes presence mainly in the cytosolic compartment of the cell. Therefore, reduction of MTT and other tetrazolium dyes depends on the cellular metabolic activity due to NAD(P)H flux. Theoretically, cells with a low metabolic activity reduce less MTT than rapidly dividing cells. Factors affecting the results including, MTT dye and DMSO affect the viability of tested bacterial cells, in addition the place where the reduction happened either intracellular or extracellular will affect the amount of reduction (Berridge and Tan, 1993).

MTT assay was used to measure the metabolic activity of biofilms in previous studies (Cheng et al., 2012a, Cheng et al., 2012b, Cheng et al., 2012c, Cheng et al., 2012d); (Antonucci et al., 2012) however, a modified criteria was used in this study to test the metabolic activity of biofilms formed over GIC disks.

4.3.2 MTT Assay Technique

- Each disk carrying the biofilm was transferred to a new 24-well plate. 500µl of MTT dye (0.5 mg/mL MTT in PBS) was added to each well and incubated for 1 h at 37°C. During this process, metabolically active bacteria reduced the MTT to purple formazan.
- After 1 h, 500 µl of dimethyl sulfoxide (DMSO) was added to each well to solubilise the formazan crystals, and the plate was incubated for 20 min at 37°C in the dark while rocking on a shaker.
- After mixing via pipetting, the disks were removed and the absorbance at 620 and 550 nm (optical density OD) was measured via a microplate reader (SpectraMax M5, Molecular Devices, Sunnvale, CA), then OD_{550} subtracted from OD_{620}. A higher absorbance is related to a higher formazan

concentration, which indicates a higher metabolic activity in the biofilm adherent on the disk (Figure 4.11).

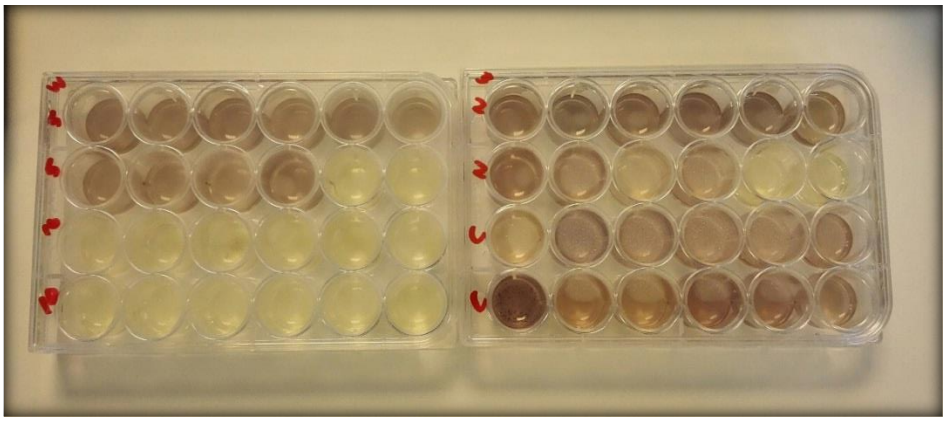


Figure 4.11 24 well plates contains MTT reagent mixed with DMSO.

Wells with purple color represent metabolic activity of bacteria while the yellow wells represent negative metabolic activity.

4.4 Harvesting the Biofilm

GIC disks were incubated with specific number of bacterial cells in DMM to allow biofilm formation over disks. After incubation each disk holding the biofilm was picked carefully and was placed in an Eppendorf filled with 1 ml of sterile PBS then the bacteria were harvested by sonication (1510E-MT, Branson Ultrasonics, Danbury, CT) (Figure 4.12) at a frequency of 40 kHz for 5 minutes, then by vortexing at the maximum speed for 30 s using a vortex mixer (Fisher, Pittsburgh, PA). The bacterial suspension thus collected was serially diluted in PBS and plated on selected agar plates.

To examine whether the sonication method successfully harvested all the bacteria from the disk surfaces, the sonicated disks were examined using Scanning Electron Microscopy (SEM).

Figure 4.12 Branson sonicator.

4.5 Microscopy

4.5.1 Light Microscope

Light microscope (IX50, Olympus, Tokyo, Japan) was used for the following functions:

- Monitoring the growth, shape, division and death of the consortia species.
- Making sure of a contamination free culture.

4.5.2 Scanning Electron Microscope (SEM)

Because of the light microscope limitations in visualising the biofilm formed on glass ionomer disks as the light cannot go throw the glass ionomer material, SEM was the best method to see the characteristics and structure of the biofilm.

Samples preparation for SEM:

- Disks were transferred to 24 well plates and impregnated in 1 ml of 4% Paraformaldehyde in 0.2M sodium buffer; the disks were left in the fixative for 2Hrs at room temperature.
- The fixative was replaced with sterile PBS for 15 minutes; this step was repeated for 5 times to wash out the remains of the fixative.
- The fixed tissue on disks was then dehydrated. Because air-drying causes collapse and shrinkage, this is commonly achieved by replacement of water in the cells with organic solvents such as ethanol or acetone, in this case a serial dehydration with 100% ethanol for three times.
- The dehydrated disks were then subjected to critical drying using a BAL-TEC CPD 030 for replacement of these solvents with a transitional fluid in this case this was liquid carbon dioxide. The carbon dioxide was finally removed while in a supercritical state, so that no gas–liquid interface is present within the sample during drying.

- The dried specimens were then sputter-coated with gold/palladium coating 15nm thick (Figure 4.13) using a Cressington 208HR sputter coater, and examined via SEM (JEOL 7400F Scanning Electron Microscope operating at an accelerating voltage SRV).

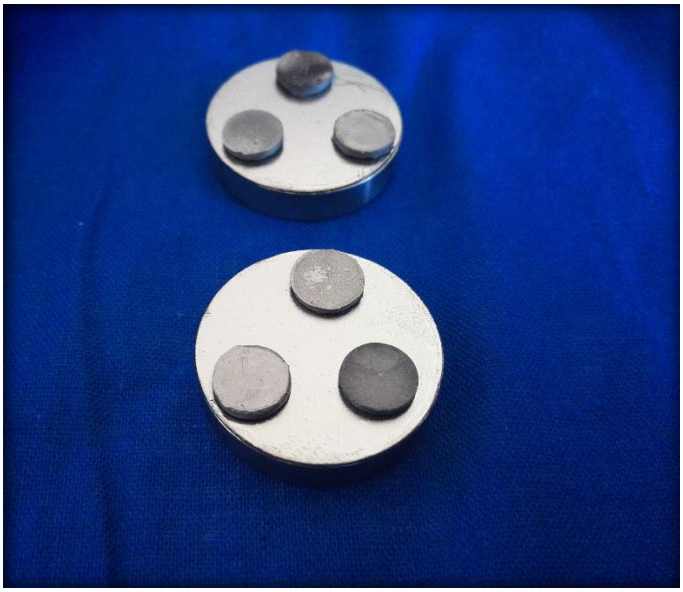


Figure 4.13 Glass ionomer disks with dried biofilm plated with 15nm Au/Pd and ready for SEM.

4.6 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a known technique in molecular biology used to amplify a single copy or a few copies of a DNA sequence across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. It is a very useful tool for diagnosis and monitoring purposes where the work is focused in amplification of specific DNA segment, like genetic diseases or bacterial organism identification (Bartlett and Stirling, 2003).

It was developed in 1983 by Kary Mullis (Bartlett and Stirling, 2003) ; (Mullis et al., 1987). Nowadays PCR is a very common tool used in clinical and research laboratories for a wide range of applications, including DNA cloning and sequencing, genes analysis, diagnosis of genetic diseases, and detection of pathogenic microorganisms which help in disease diagnosis (Ninfa and Ballou, 2004).

4.6.1 The Principle of PCR

The main principal is dependent on thermal cycling (cycles of repeated heating and cooling) of the reaction, which allows DNA melting and reaction enzymes can replicate the specific gene.

Primers are short DNA fragments that contain a complementary sequence to the specific region to be amplified by the DNA polymerase. They are the key PCR components which enables the reaction to be specific and consistent.

DNA polymerase is heat stable, for example Taq polymerase, which works by enzymatically assembling a new DNA strand from DNA building-blocks (nucleotides) of the single stranded DNA after melting (DNA template).

Most PCR methods typically amplify DNA fragments of between 0.1 and 10 kilo base pairs (kbp), although some techniques allow for amplification of fragments up to 40 kbp in size (Cheng et al., 1994). The amount of amplified product is determined by the

available substrates in the reaction, which become limiting as the reaction progresses (Carr and Moore, 2012).

4.6.2 PCR Components

A basic PCR set up requires several components and reagents. These components include:

- DNA template that includes the target DNA region.
- Two primers with complementary ends to DNA target region. Primers are custom made in laboratories to that specific sequence to be amplified.
- DNA polymerase (e.g. Taq polymerase) because DNA primers can't attach to a DNA strand and elongate on its own. It should also be heat resistant, so that it can withstand the denaturation process.
- Deoxynucleoside triphosphates (dNTPs, sometimes called "deoxynucleotide triphosphates"; nucleotides containing triphosphate groups), the building-blocks from which the DNA polymerase synthesizes a new DNA strand.
- Buffer solution which provides the optimum chemical environment for the reaction and stabilises the DNA polymerase.

4.6.3 Procedure

As mentioned before PCR consists of a series of 20–40 repeated temperature cycling, called cycles. Those cycles are delivered using thermal cycler device (Figure 4.14).

Cycling is often preceded by a single temperature step at a high temperature > 90 °C, called preheating, and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (Tm) of the primers (Rychlik et al., 1990).

Each cycle consists of the following steps:

- **Initialisation step**: By heating the reaction to a temperature of 94–96 °C (or 98 °C if extremely thermostable polymerases are used), which is held for 1–9 minutes.

- **Denaturation step**: This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

- **Annealing step**: The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. This temperature must be low enough to allow for hybridisation of the primer to the strand, but high enough for the hybridisation to be specific, i.e., the primer should only bind to a perfectly complementary part of the template. If the temperature is too low, the primer could bind imperfectly. If it is too high, the primer might not bind. Typically, the annealing temperature is about 3–5 °C below the Tm of the primers used. Stable DNA–DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation. It is very vital to determine the annealing temperature in PCR. This is because in PCR, efficiency and specificity are affected by the annealing temperature. An incorrect annealing temperature will cause an error in the test.

- **Extension/elongation step**: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–

80 °C (Chien et al., 1976, Lawyer et al., 1993), and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesises a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to amplify. As a rule-of-thumb, at its optimum temperature, the DNA polymerase polymerises a thousand bases per minute. Under optimum conditions, i.e. if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

The processes of denaturation, annealing and elongation constitute one cycle. Multiple cycles are required to amplify DNA.

Figure 4.14 Thermal cycler for PCR (white arrow).

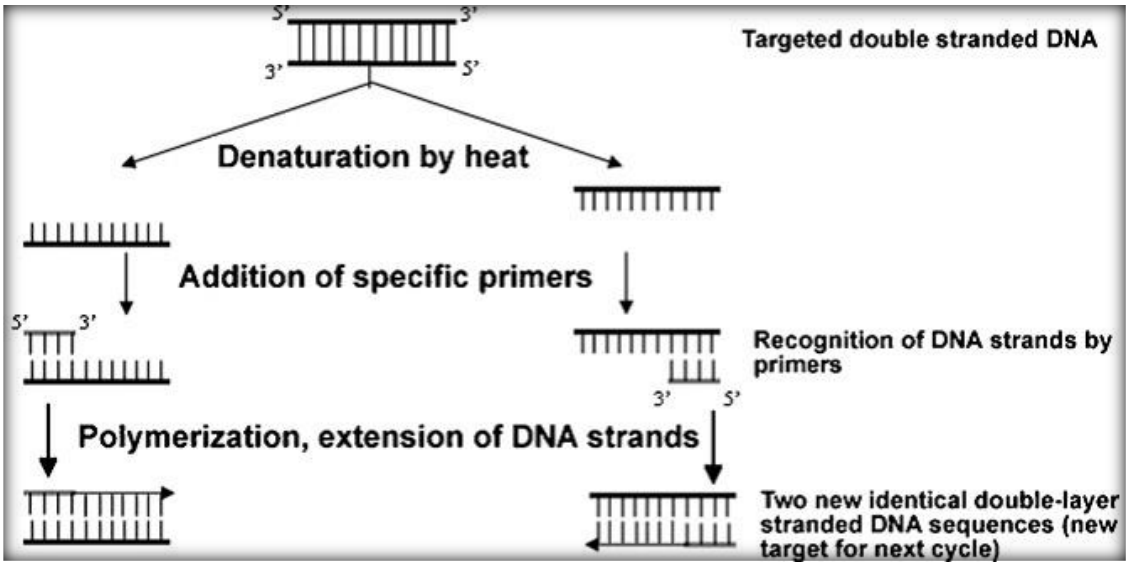


Figure 4.15 PCR reaction stages for a piece of double stranded DNA. (Higuchi et al., 1992).

4.7 Gel Electrophoresis

Gel electrophoresis is a technique used to separate and analyse macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge. It is used in clinical chemistry to separate proteins by charge and/or size and in biochemistry and molecular biology to separate a mixed population of DNA and RNA fragments by length, to estimate the size of DNA and RNA fragments or to separate proteins by charge (Kryndushkin et al., 2003).

4.7.1 The Principle

DNA or RNA molecules are separated by applying an electric current field so the negatively charged molecules will move through a matrix of a gel. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving. Proteins are separated by charge in agarose because the pores of the gel are too large to sieve proteins (Rychlik et al., 1990).

The gel works by retarding molecules from moving through it depending upon the size and charge of those molecules.

DNA gel electrophoresis is usually performed for analytical purposes, often after amplification of DNA via PCR, but may be used as a preparative technique prior to use of other methods such as DNA cloning and sequencing.

4.7.2 Agarose Gel Electrophoresis

Agarose gels are easily cast and handled compared to other matrices, because the gel setting is a physical rather than chemical change. Samples are also easily recovered. After the experiment is finished, the resulting gel can be stored in a plastic bag in a refrigerator.

Most agarose gels are made with between 0.7% (good separation or resolution of large 5–10kb DNA fragments) and 2% (good resolution for small 0.2–1kb fragments) agarose dissolved in electrophoresis buffer. Up to 3% can be used for separating very tiny fragments but a vertical polyacrylamide gel is more appropriate in this case. Low percentage gels are very weak and may break when you try to lift them. High percentage gels are often brittle and do not set evenly. 1% gels are common for many applications.

4.7.3 Buffers

Buffers in gel electrophoresis are used to provide ions that carry a current and to maintain the pH at a relatively constant value. These buffers have plenty of ions in them, which is necessary for the passage of electricity through them. There are a number of buffers used for electrophoresis. The most common being, for nucleic acids Tris/Acetate/EDTA (TAE), Tris/Borate/EDTA (TBE).

4.7.3.1 TBE Buffer Preparation Protocol

5X TBE (1 litre)

- 54 g Tris and 27.5 g boric acid in 800 ml distilled water were dissolved using magnetic stirrer at room temperature till it was all dissolved.
- 20 ml 0.5 M Na_2EDTA (pH 8.0) were added.
- Volume was adjusted to 1 Litre.
- Then it was stored at room temperature till it was all used.
- 1:10 dilution of this solution to make 0.5x TBE was used to make the agarose gel.

4.7.3.2 Preparation of a 1% Agarose Gel (Figure 4.16).

- The gel casting tray (Figure 4.17) was rinsed and dried with 95% ethanol.
- The level of the comb was adjusted so that it rested evenly with a few mm of space between teeth and the tray; this allowed the wells to form in the agarose.
- The 1% agarose had already been prepared (1 g agarose powder per 100 mL 0.5x TBE), and melted using microwave till it was fully dissolved.
- 10µl of Cyber/Green dye were added to the solution and then the agarose was poured into the casting tray.
- The gel was allowed to solidify at room temperature (about 1 hour); then the comb was removed.

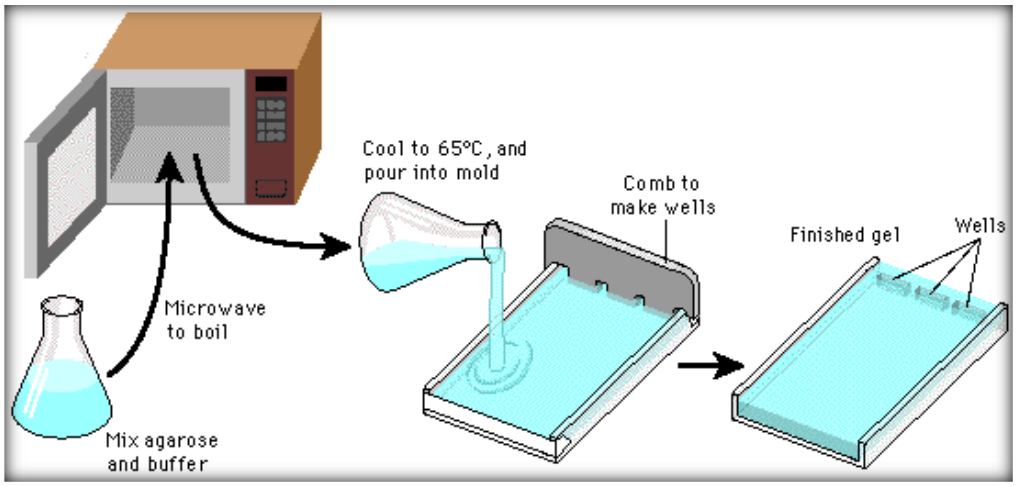


Figure 4.16 Agarose gel preparation (Adapted from Pearson Education, Inc.)

4.7.4 Loading the Agarose Gel with PCR product and Running PCR

- The casting try was inserted (with the gel in it) into the electrophoresis chamber with the wells closest to the negative (black) electrode. DNA is negatively charged. During electrophoresis, it will migrate from the negative to the positive electrode.
- Gradually 5XTBE (Tris-Borate-EDTA; electrophoresis buffer) was added to the chamber until the buffer just covers the top of the gel.
- 10µl of each PCR product was loaded in each well, taking care not to puncture the well bottoms.
- 5µl of Hyperladder® marker was inserted in the last well, which is a DNA ladder containing DNA fragments of defined length for sizing the bands in the experimental PCRs.
- The lid then was attach, and the cords make were correctly plugged into the power supply (red to red, black to black).
- The power supply was plugged in and the voltage knob was adjusted to 70 volts.
- The Electrophorese was run for an hour or until the dye has migrated to within ¾ of the positive electrode end of the gel.
- The power supply was shut off and the gel casting tray was lifted from the chamber and the gel was gently slide over a UV trans-illuminator (Figure 4.18).
- The plastic cover over the trans-illuminator was closed for eye protection then a photo of the gel was taken after turning the UV light on using a digital camera linked to the trans-illuminator (Figure 4.18).

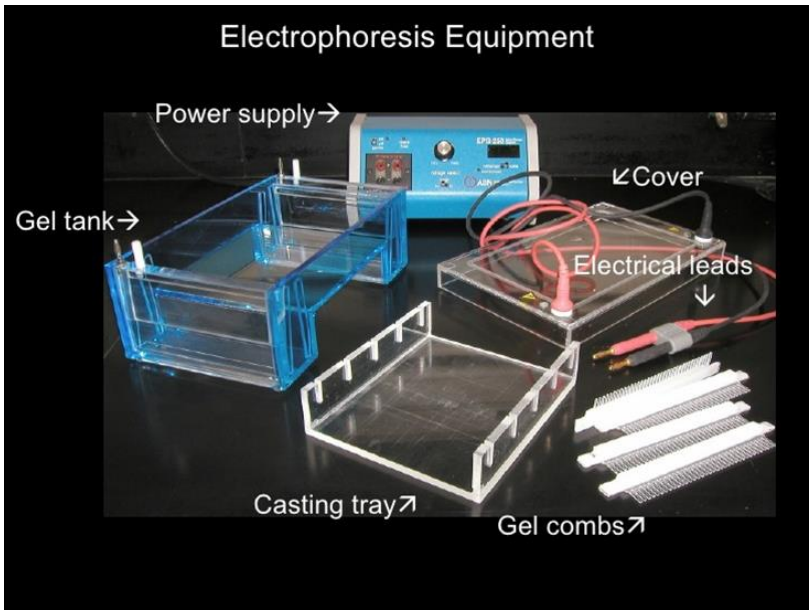


Figure 4.17 Gel electrophoresis equipment (Adapted from Pearson Education, Inc.).

Figure 4.18 UV trans-illuminator (1) with over-mounted Kodak camera (2) attached to PC monitor (3)

4.8 Polyvinyl Alcohol and Silver Ion Solutions

Silver citrate ($Ag_3C_6H5O_7$) is a white substance that has a limited water solubility. Under the normal physicochemical conditions, 1 part of silver citrate is soluble in 3500 parts of water, which corresponds to 285 ppm of Ag^+ ions in the solution (Arata, 2001).

The maximum concentration of silver ions (Ag^+) in a saturated solution of silver citrate in water can be estimated at about 0.3 g/L (2.8×10^{-3} mol/L), which is in agreement with the data in the literature (Arata, 2001). However, Djoki´c (Djokic, 2008) has found that silver citrate can quite successfully be dissolved in the aqueous solutions of citric acid.

In addition, if citric acid concentration increased, for the fixed amount of silver citrate, that leads to an increase in the concentration of silver ions in solution. These results strongly suggest that an increase in the concentration of citric acid in solution leads to an increase in the solubility of silver citrate (Djokic, 2008).

4.8.1 Difference between Silver Nitrate and Silver Citrate Dissolution in Acids

Dissolution of silver nitrate ($AgNO_3$) into citric acid ($H_3C_6H_5O_7$)solution or other hydroxycarboxylic acids does not lead to precipitation of silver citrate ($Ag_3C_6H_5O_7$) or other hydroxycarboxylates (e.g., malate, tartrate, lactate, etc.) (Bailar and Trotman-Dickenson, 1973).

In the case of silver nitrate and citric acid, this behaviour can be explained by the following reaction:

$$\mathbf{3AgNO_3 + H_3C_6H_5O_7 \longleftrightarrow Ag_3C_6H_5O_7 \downarrow +3HNO_3} \quad \mathbf{(1)}$$

Due to the dissolution of silver citrate in HNO_3, the precipitation of $Ag_3C_6H_5O_7$ does not occur. Therefore, it can be concluded that the equilibrium of the reaction (1) is shifted to the left (in the reverse direction).

Silver citrate dissociates in aqueous solutions according to the following reaction:

$$Ag_3C_6H_5O_7 \longleftrightarrow 3Ag^+ + C_6H_5O_7 \quad (2)$$

Consequently, the equation for the solubility product of silver citrate can be written as:

$$Ksp(Ag_3C_6H_5O_7) = [Ag]^+{}_3 + [C_6H_5O_7]$$

Generally, in a solution with a constant concentration of citric acid, an increase in the amount of silver citrate leads to an increase in the concentration of silver ions in solution. However, the maximum amount of silver citrate which can be dissolved depends on the citric acid concentration. For example, the maximum amount of silver citrate soluble in a 50 cm^3 of 0.5 M citric acid solution is estimated at about 0.4 g, which corresponds to the Ag^+ ion concentration of about 5 g/L. A further increase in the amount of silver citrate in a 0.5 M citric acid solution does not lead to an increase in the concentration of Ag^+ ions, suggesting that dissolution does not take place and that saturation is achieved.

However, a rise in the concentration of citric acid leads to an increase in the amount of silver citrate which can be dissolved. In concentrated citric acid solutions (3 mol/L to 4 mol/L), which corresponds to about 576 to 768 g/L, the maximum concentration of silver ions is estimated to about 22–25 g/L (0.204–0.232 mol/L). These concentrations of silver ions correspond to the amount of silver citrate in the range from 34.85 g/L to 39.60 g/L. A further addition of silver citrate to the concentrated citric acid solutions does not lead to dissolution, suggesting that saturation is achieved. On the other hand, the preparation of citric acid solutions with concentrations more than 4 mol/L faces experimental difficulties, even with heating, since crystallisation of citric acid occurs due to saturation.

How can the dissolution of silver citrate in citric acid solutions be described? Citric acid, $Ag_3C_6H_5O_7$, contains three carboxylic groups (–COOH) and, consequently, three hydrogen ions can be replaced with metal ions, as it is observed in the precipitation of silver citrate, $Ag_3C_6H_5O_7$. When one or two hydrogen ions from the carboxylic groups of citric acid are replaced with Ag^+ ions $AgH_2C_6H_5O_7$ or $Ag_2HC_6H_5O_7$ should be produced, respectively. The formation of these compounds can be described with the following reactions:

$$\mathbf{Ag_3C_6H_5O_7 \downarrow + 2H_3C_6H_5O_7 \longrightarrow 3AgH_2C_6H_5O_7} \qquad \mathbf{(3)}$$

$$\mathbf{2\ Ag_3C_6H_5O_7 \downarrow + H_3C_6H_5O_7 \longrightarrow 3Ag_2HC_6H_5O_7} \qquad \mathbf{(4)}$$

Based on the stoichiometry of the reactions (3) and (4) and an assumption that $AgH_2C_6H_5O_7$ or $Ag_2HC_6H_5O_7$ is soluble in water, it can easily be calculated that the amounts of citric acid for the dissolution of 100 g of $Ag_3C_6H_5O_7$ (0.195 mol) are, respectively, 74.89 g (0.384 mol) for the reaction (1) and 18.72 g (0.0975 mol) for the reaction (2).

However, it was not the case as it was experimentally found that significantly larger amounts of citric acid were required for the dissolution of $Ag_3C_6H_5O_7$ than those calculated on the basis of the stoichiometry of the reactions (3) and (4). Consequently, if $AgH_2C_6H_5O_7$ and $Ag_2HC_6H_5O_7$ were soluble in water, it is unlikely that the dissolution of silver citrate proceeds via reactions (3) and (4). On the other hand, chemical analysis of "undissolved" precipitate found that it (precipitate) contained about 63% Ag, which corresponds to $Ag_3C_6H_5O_7$ and not to $AgH_2C_6H_5O_7$ or $Ag_2HC_6H_5O_7$. Considering the fact that significantly larger amounts of citric acid are required for the dissolution of silver citrate, it is reasonable to assume, based on the silver analysis and stoichiometry of the

chemical equations, that citric acid produces complexes with silver citrate according to the following reaction:

$$Ag_3C_6H_5O_7 + nH_3C_6H_5O_7 \longleftrightarrow [Ag_3(C_6H_5O_7)_{n+1}]^{3n-} + 3nH^+ \quad (5)$$

The complexes with a general formula stated as $[Ag_3(C_6H_5O_7)_{n+1}]^{3n-}$, where n is an integer, that is, n = 1, 2, 3...., are expected to be soluble and stable in water. On the other hand, the reaction (5) is reversible, and depends on both amounts of silver citrate and citric acid concentration.

4.8.2 PVA Solution Preparation (used to stabilise silver ions in solution)

4.8.2.1 Materials

- Polyvinyl Alcohol 15,000 MW (DBH Chemical Ltd, Poole England)
- Deionised water.

4.8.2.2 Method of Preparation

- 500 ml of water were heated to ~ 90°C and 5g PVA were added slowly, with stirring (using a hot plate and a magnetic stirrer).
- Stirring and heating was continuous for a further ~ ½ hour, then it was left overnight to cool in 4°C fridge.
- It was found that it was convenient to weigh out the PVA by adding it to a small beaker, which was first placed on the balance and restored to zero weight. This avoids spillage of PVA, and it is then convenient to sprinkle the PVA into the water during the heating/stirring process.
- A watch glass should be placed over the beaker to reduce loss of water by evaporation. At the end of the operation pure water should be added to restore the volume of liquid to its original value.
- This resulted in a concentrated PVA stock solution of 10 mg/ml.

4.8.3 Silver Citrate/Citric Acid Complexes Preparation. (The resulted solutions presented in Figure 4.19 were used as antimicrobial additives to GIC in this project)

4.8.3.1 (5) mg/ml Ag^+ (8 mg/ml silver citrate)

4.8.3.1.1 Materials

- Silver citrate hydrate 512.70 MW (Sigma-Aldrich, Germany).
- Citric acid monohydrate 210.14 MW (Sigma-Aldrich, Germany).
- Deionised water.

4.8.3.1.2 Method of preparation

- 2.25g of citric acid were added to 50ml of deionised water to prepare 0.5M citric acid solution.
- 0.4 g of silver citrate were added to 50 ml of .5 M citric acid and then it was heated to dissolve.

4.8.3.2 (22 and 13) mg/ml Ag^+ (34.85 mg/ml silver citrate)

4.8.3.2.1 Materials

- Silver citrate hydrate 512.70 MW (Sigma-Aldrich, Germany).
- Citric acid monohydrate 210.14 MW (Sigma-Aldrich, Germany).
- Deionised water.

4.8.3.2.2 Method of Preparation

- 20 ml of water were added and 42.028 g of citric acid then was heated to dissolve this yield 50 ml of 4M citric acid. Some water might be needed to top it up to 50ml.
- 50 ml of 4M citric acid and 1.74g of silver citrate then were added together and the solution was heated to dissolve which resulted in 22 mg/ml Ag^+ solution.

- This solution was not stable; recrystallisation was noticed on the second day that resulted in diluting it to reach 13mg/ml by adding water. That reduced the molarity of citric acid to 2.7 M.

4.8.3.3 (10) mg/ml Silver Citrate/PVA Solution

4.8.3.3.1 Materials

- Silver citrate hydrate 512.70 MW (Sigma-Aldrich, Germany).
- Citric acid monohydrate 210.14 MW (Sigma-Aldrich, Germany).
- Deionised water.

4.8.3.3.2 Method of preparation

- 2ml of stock PVA solution were added to 18ml of distilled water, then the mixture was heated to 80 °C.
- 9ml of 22mg/ml solution were added dropwise and mixed with a magnetic stir for 10 minutes, the resulting solution was 20ml of 10mg/ml Ag^{+} with PVA. PVA concentration in that solution was 5 mg/ml.

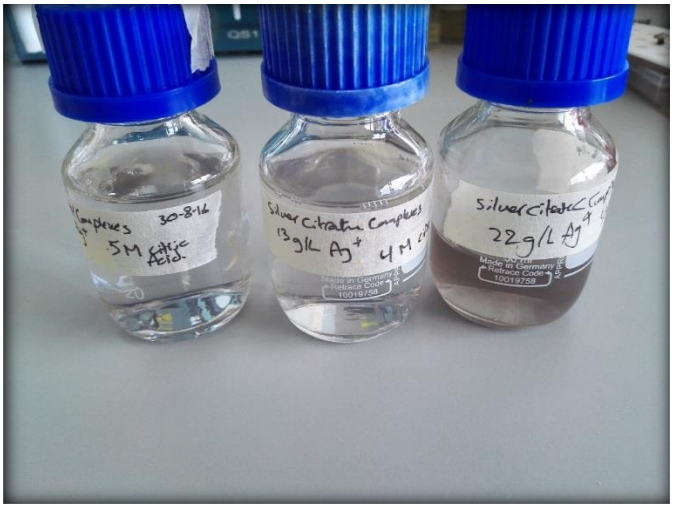
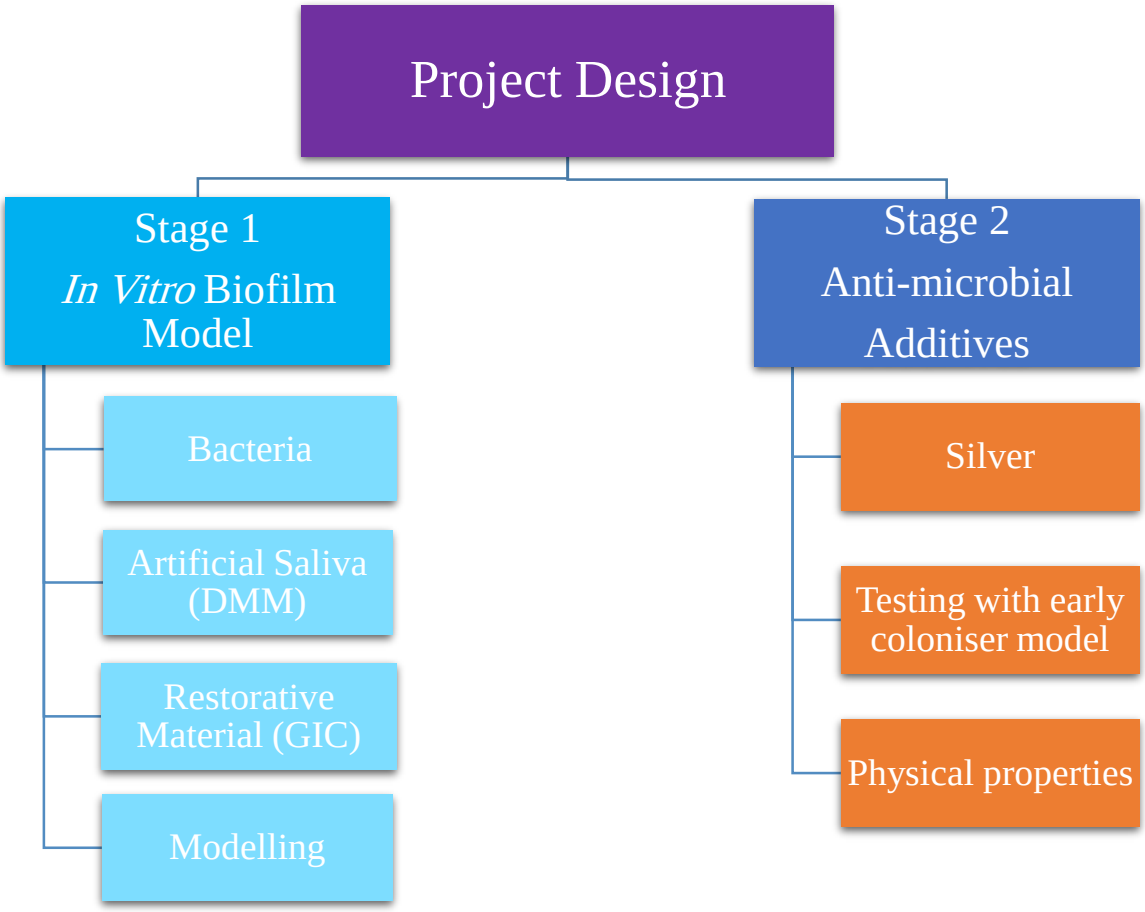


Figure 4.19 Silver citrate/ citric acid solutions**;** from right to left 22 mg/ml, 13 mg/ml and 5 mg/ml.

Chapter 5 EXPERIMENTAL PART I (Development of *in vitro* Biofilm Model System)

The *in vitro* model consisted of four main building blocks as shown in the following flowchart; oral bacteria, artificial saliva, restorative material (in this project GIC was used) and lastly modelling all the building blocks together through a set of conditions to make the model. Then in chapter 6, after achievement of a consistent and reproducible *in vitro* model, the model was used to test the effect of silver addition to GIC on the colonisation of the model species, furthermore the effect of silver addition on the physical properties of GIC was tested. In this chapter a series of 10 experiments were conducted to validate and build the *in vitro* biofilm model.



5.1 Test Single Species Growth of Consortium Species in DMM

After selection of eight species and then sub-selection of the three early colonisers (*S. oralis S. mutans* and *N. subflava*) (section 4.1.1.1), the next stage was to test their growth in the selected artificial saliva (DMM) by comparing it to their growth in commercially available media i.e. Columbia Broth (CB), Brain Heart Infusion (BHI) etc...

The aim was to assess if DMM is suitable to support the growth of those species in comparison to a commercially well know media, consequently to identify the optimum conditions for each species growth and to assess if any modification was needed to the original recipe.

5.1.1 Materials and Methods

5.1.1.1 Bacterial Species

S. mutans
S. oralis
E. faecalis
A. actinomycetemcomitans
N. subflava
F. nucleatum.
T. forsythia
V. parvula

5.1.1.2 Growth Medium: DMM, CB, BHI.

5.1.1.3 Culturing Methods and Growth Conditions

For *S. mutans, S. oralis, E. faecalis, N. subflava;*

Species were streaked out on labelled CB plates using the method described in section (4.1.1.3.2.1). Then they were incubated in an aerobic incubator at 37°C for 24Hrs. After

growing on CB plates, a single colony from each species was picked up from CB plates using sterile loop and then it was mixed with 10ml of sterile CB in a sterile flask.

The labelled flasks were covered with sterile foil then transferred to 37°C water bath shaker at speed 200RPM (Grant instruments, Cambridge, UK). After 24hours a 100µl of each culture were mixed with 900µl of deionised water (OD_{600}) in a cuvette (70µl UV-cuvette micro, dimensions 12.5×12.5×45 mm, center height 8.5 mm, Brand, Germany) to measure its optical density at 600nm wavelength using spectrophotometer (Eppendorf, Hamburg, Germany).

Another culture at an OD_{600} 0.05 was made for each species but using 10ml of DMM as growth medium. That was done by inoculation of specific amount of the overnight culture (after 24Hrs incubation) depending on the OD_{600} measured using formula number 1, then flasks were left shaking in the water bath.

(OD_{600} needed/ measured OD_{600}) × Final volume = volume for inoculation… (1).

OD_{600} for each culture was measured every 30 minutes; at that point 5µl were taken each time to be checked with a light microscope to assure growth and exclude contamination.

For *F. nucleatum* and *V. parvula* and *T. forsythia* the growth was under anaerobic conditions in an anaerobic cabinet (10% CO_2, 10% H_2, 80% N_2, and 37°C). While *A. actinomycetemcomitans* is capnophilic which means it thrives in the presence of high concentrations of carbon dioxide (CO_2), it was grown in jars in an atmosphere of 5% CO_2 and 37°C.

The previous growth and culturing methods were used to prepare broth cultures for all the experiments in this project unless otherwise stated.

5.1.2 Results

Microscopically, shape, distinctive features, mobility, chaining, and clumping for each species was compared between species grown in CB and DMM. As shown in (Figure 5.1) all the species showed their expected features in DMM.

Modifications to the original recipe were needed to optimise the growth of some species like *F. nucleatum*, *V. parvula* and *T. forsythia* which are considered to be fastidious, special requirement were needed for their growth (Rocas et al., 2001).

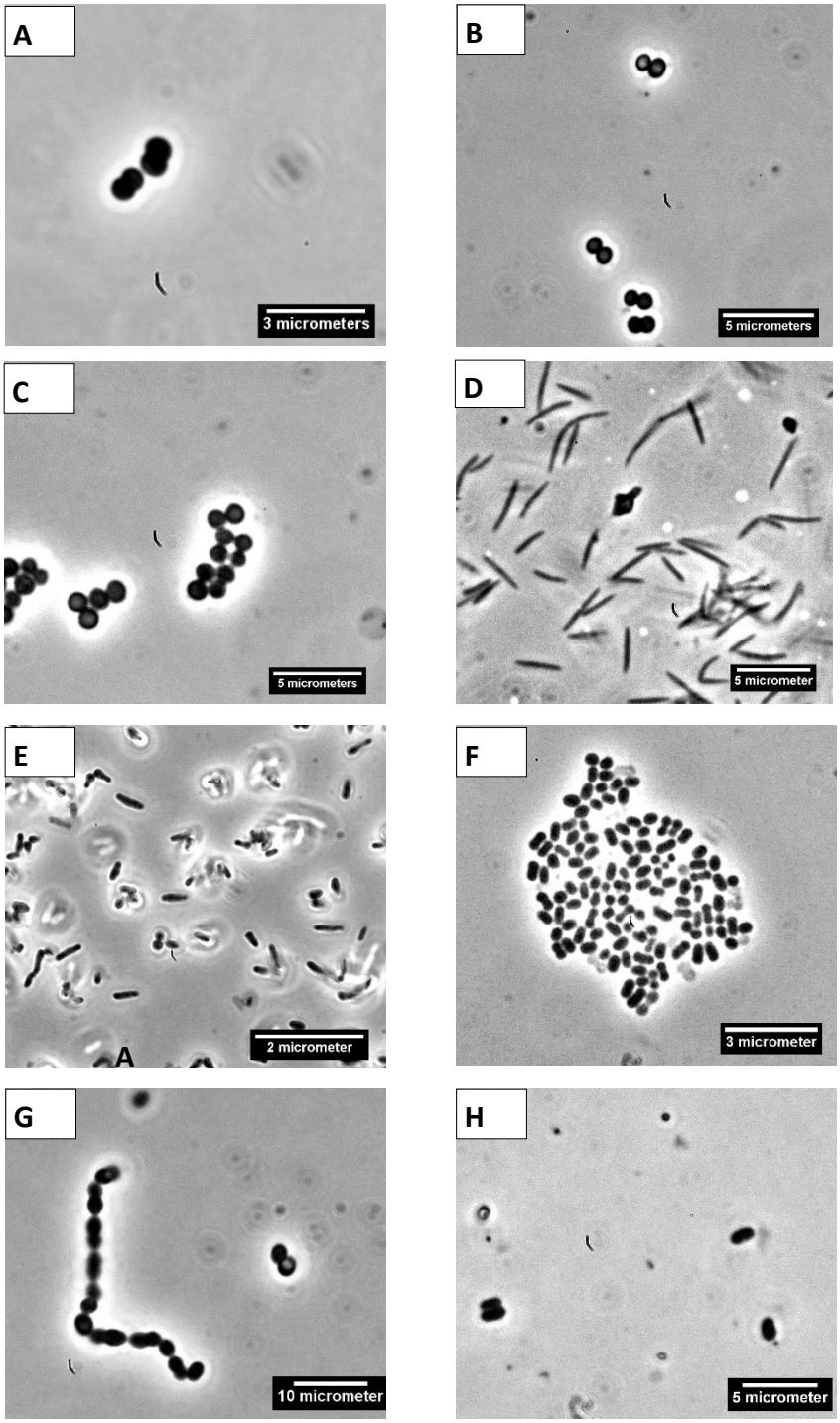


Figure 5.1 Light microscopic images of consortium bacterial species growing in DMM.

A, *N. subflava***. B**, *S. mutans***. C**, *S. oralis*. **D**, *F. nucleatum*. **E**,

A. actinomycetemcomitans. **F**, *V. parvula***. G**, *E. faecalis***. H**, *T. forsythia.*

5.1.3 Discussion

Generally, evaluation of bacterial growth in broth culture could be conducted microscopically as a subjective method, or monitoring OD_{600} reading over a period of time and that reflects the species doubling time and speed of growth. Bacteria grow and multiply by binary division so theoretically they double in number at each specific period of time.

Each species has its own distinctive features i.e. cocci, diplococci, spindle shape, chaining, clumping etc… and also has a specific cell division speed.

In this experiment the general subjective feature for each species grown in DMM was the same compared to their growth in CB. The expected shape and behavior for each species resulted from their growth in DMM as shown in (Figure 5.1). *N. subflava* is Gram-negative diplococcus. Whereas *S. mutans* and *S. oralis* are Gram-positive streptococci, in addition to the ability to form chains and clumps. However, *F. nucleatum* is rod-shaped Gram-negative, *A. actinomycetemcomitans* is Gram-negative coccobacillus. *V.parvula* is Gram-negative cocci. *E. faecalis* Gram-positive cocci and can form small chains. *T. forsythia* Gram-negative pleomorphic in shape.

T. forsythia is a fastidious microorganism, and it is a member of the red complex species; *P. gingivalis*, *T. forsythia* and *T. denticola*, in which they considered the founders of periodontal disease (Mahajan et al., 2013). They are difficult to cultivate and needs specific growth requirements (Aruni et al., 2015). In this study a specific rich medium TF broth was used to cultivate *T. forsythia*, TF broth is a Brain Heart Infusion broth supplemented with 5µg/ml menadione, 0.001% NAM, 0.1 L-cysteine, 5% fetal bovine serum (Wyss, 1989, Wyss, 1992, Megson et al., 2015). In addition, 100µl 0.001% NAM (N- Acetylmuramic Acid) were spread over Blood agar plates and also NAM was added to DMM to enhance the growth of *T. forsythia*. This insight was not mentioned in the

original recipe, because they claimed that the resulting microcosm biofilm using DMM was closer to the natural oral biofilm without any DMM modifications (Wong and Sissons, 2001). However, it is worth assuming that they may underestimate the presence of *T. forsythia* which is a keystone pathogen in periodontal diseases, this related to the superior advantage of consortium biofilms over microcosm where the later has less detailed and consistent biofilms compared to consortium. *T. forsythia* is a slow growing bacteria; four days were needed to have growth in both NAM modified DMM and TF broth. Almost same growth rate for *V. parvula*, while *F. nucleatum*, they needed 2 days in DMM and CB.

From all previous observations, DMM as a growth medium was capable of supporting the growth of the model species either aerobically or anaerobically up to 48 hours. The expected shape, size and pattern of growth for each species was observed. Modifications to DMM were needed to support the fastidious micro-organisms like *T. forsythia*, *F. faecalis* and *V. parvula*. Consequently, the behavior and growth conditions of each species in DMM was determined, that helped in designing the model aiming toward mixed community, taking into consideration their growth methodology, doubling times and special requirements in artificial saliva.

Three of these consortium species were choose to be studied in further detail and to be the foundation for the whole model, next section for further details.

5.2 Comparing the Growth of Early Coloniser Model Species between DMM and CB

5.2.1 Materials and Methods

5.2.1.1 Bacterial Species

Three species were selected out of the original consortium, because they were considered as early colonisers in the natural oral biofilm; *S. oralis*, *S. mutans* and *N. subflava*. Streptococci are considered to be the main group of early colonisers in the oral biofilm, making up over 80% of the initial biofilm especially Gram-positive facultative anaerobic (Marcotte and Lavoie, 1998). They interact with other primary colonisers and attach to the tooth surface, determining the composition of late colonisers in the oral biofilm, and impacting the health or disease status of the host (Hajishengallis et al., 2012, Kolenbrander et al., 2010). *S. oralis* which attached directly to the acquired pellicle as mentioned earlier in literature review section (Kolenbrander et al., 2010). However, *N. subflava* is also considered a Gram-negative pioneer coloniser (Kolenbrander, 1995). In addition, *S. mutans*, a Gram-positive facultative anaerobic bacteria and considered to be part of early colonisers, despite the fact that it is a keystone in dental caries pathogenesis which was extensively studied in the literature (Marcotte and Lavoie, 1998); (Bowen and Koo, 2011). Those three species (*S. oralis*, *N. subflava* and *S. mutans*) considered to form an early coloniser model which was studied in detail in this project.

5.2.1.2 Growth Medium; DMM and CB**.**

5.2.1.3 Culturing Methods and Growth Conditions

An overnight culture (after 24Hrs incubation) for each species was prepared in CB aerobically using the protocol described in section (5.1.1.3) then they were diluted back to OD_{600} 0.05 in DMM and CB and they were left shaking in water bath 37ºC aerobically at 200RPM.

Growth for each culture was monitored by measuring OD_{600} reading every 30 minutes up to 3Hrs then after 24 and 48Hrs while they were shaking in a 37ºC water bath. At each time OD_{600} measurement was taken, 5µl from each sample were spread over glass-slap and were checked using light microscope to ensure growth and exclude contamination.

OD_{600} readings were plotted against time and a growth curve resulted for each species in the examined growth media.

5.2.2 Results

Growth curve for *S. mutans*, *S. oralis* and *N. subflava* in DMM compared to CB are presented in Figures 5.2 to 5.4. Growth curves represent the growth pattern, speed and doubling time of each species either in CB or DMM.

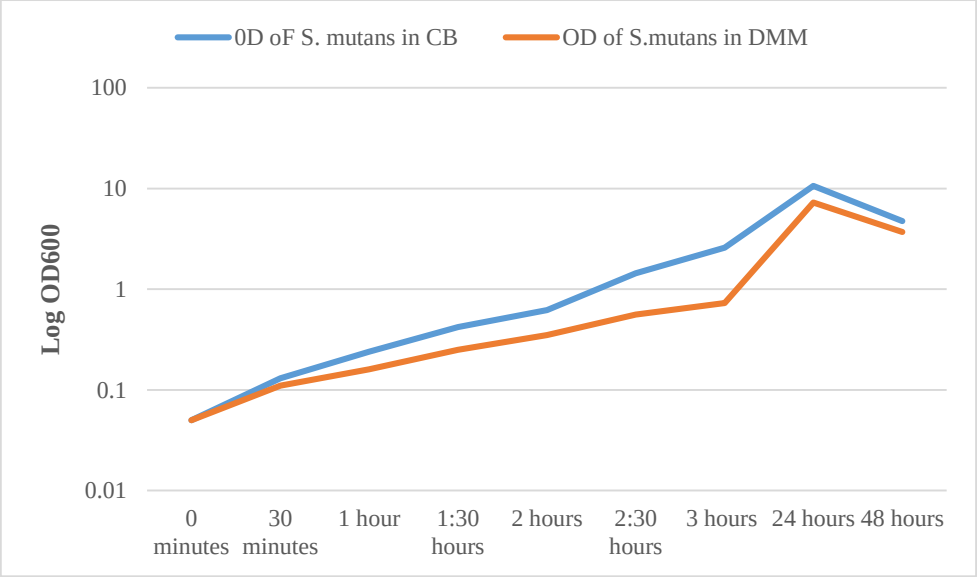


Figure 5.2 Graph represent growth curves of *S. mutans* in CB broth compared to DMM. Showing that it grows in both media.

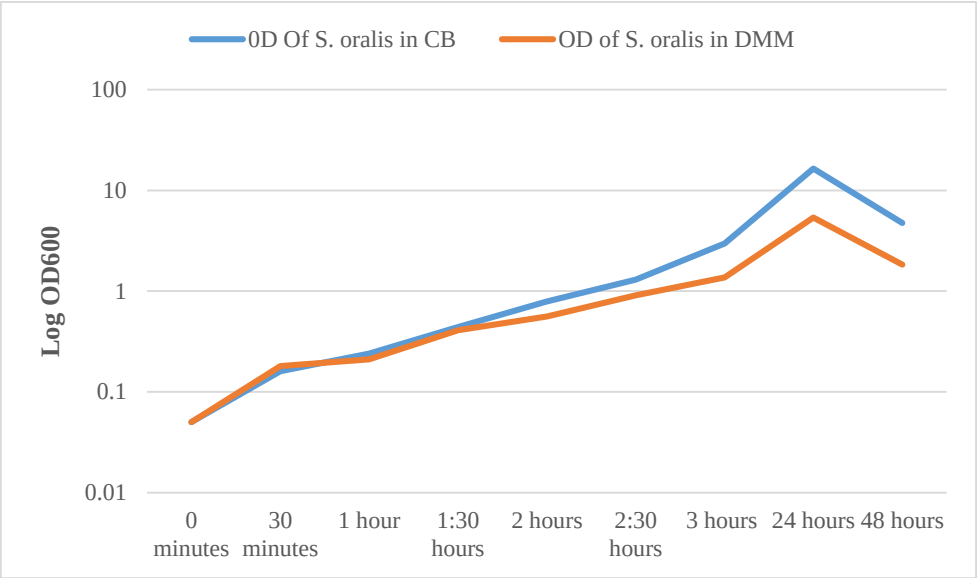


Figure 5.3 Graph represent growth curves of *S. oralis* in CB broth compared to DMM. Showing that it grows in both media.

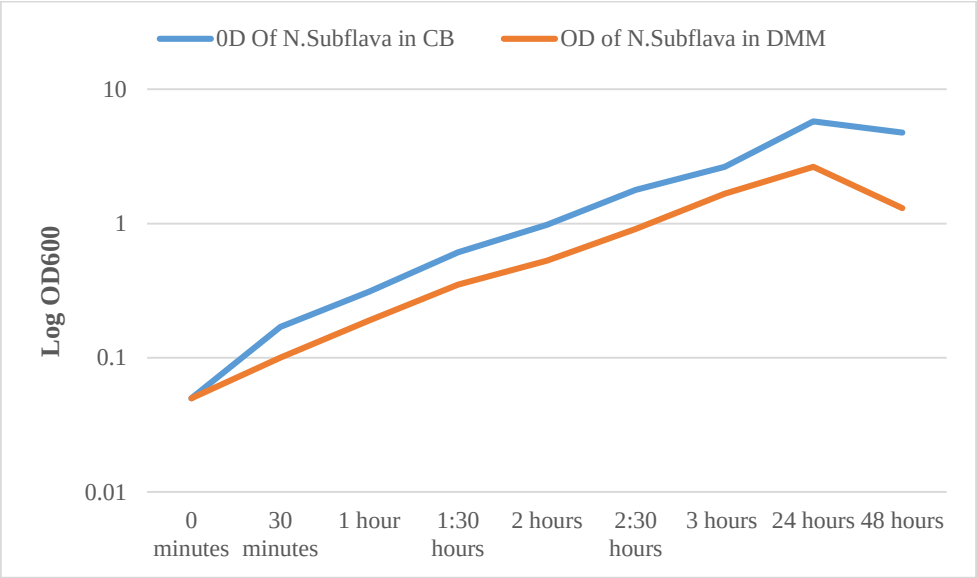


Figure 5.4 Graph represent growth curves of *N. subflava* in CB broth compared to DMM. Showing that it grows in both media.

5.2.3 Discussion

Artificial saliva should be nutritive and host features that favors bacterial cultivation in a similar way that the natural saliva does. Proteins and glycoproteins in saliva are the major nutrients and energy source for the dental plaque bacteria (Smith and Beighton, 1986; Bradshaw et al., 1994). CB is a rich commercial bacterial growth medium that support growth of a wide variety of bacterial species. Its protein components including peptones and yeast extracts (section 4.1.1.3.2.2), the enzymatic digest of Casein is about 5 g in each litre of CB. In comparison, DMM has an equivalent amino acid mixture based on 5 g/l casein and it was considered the major environmental simplification compared with saliva (Wong and Sissons, 2001). The advantages of using amino acids equivalent to casein rather that its enzymatic digest are; first casein is a major dietary protein, it and its hydrolysates are commercially available, second the growth on intact proteins, peptone mixtures and component amino acids not requiring peptide hydrolysis. Lastly, amino acids concentrations could be measured and compared between cultures (Sissons et al., 1997).

Mucin plays a critical role in acquired pellicle formation and that is considered the first step in natural biofilm formation (Gibbins et al., 2014, Hannig and Joiner, 2006). Consequently, in *in vitro* biofilms modelling important salivary components like mucin and cystatins which are provided by DMM are really important to have biofilms which are close to that formed in the oral cavity (Wong and Sissons, 2001).

BMM, a complex mucin-containing medium (Glenister et al., 1988), has been widely used for *in vitro* studies of oral bacteria. Although BMM is a standard culture medium for oral microbial studies, it contains large quantities of various unknown peptides from the manufacture of yeast extract and peptone mixtures, giving inconsistent and usually

unknown concentrations of specific peptides, vitamins and ions between batches (Wong and Sissons, 2001).

It is essential to use a chemically defined medium in which known changes can be made to individual components, for example, to study factors regulating plaque growth and mineralisation.

In this experiment, the growth of three species aerobically at 37ºC in DMM was similar to but slower than in CB especially in the case of *N. subflava* which was on average 42% slower than CB, (Figure 5.4). Wong and Sissons stated the same finding when they used DMM to make a microcosm model (Wong and Sissons, 2001) but percentages were not calculated. Doubling times for *S. mutans* and *S. oralis* in DMM aerobically was 35% and 27% respectively slower on average than in CB, (Figure 5.2 and 5.3).

Although the growth of early coloniser model species was slower in DMM, they were dividing and growing following the normal growth curves pattern. Consequently, DMM is considered a rich saliva analogue especially in the presence of mucin and cystatins which favours biofilm formation in a more natural way. In addition, DMM contains a defined component superior to other artificial saliva recipes for example BMM (Wong and Sissons, 2001) allows changes to be made to its individual components, for example, to study factors regulating plaque growth and mineralisation.

5.3 The Effect of DMM Aging and Storage Time on the Growth of Early Coloniser Species

DMM as saliva analogue contains a wide range of proteins, vitamins and growth factors. However, these constituents may degrade with time which may affect the reproducibility of early coloniser model species growth. To test the effect of storage time of DMM on the growth of early colonisers the following experiment was conducted.

5.3.1 Materials and Methods

Same as in previous experiment, but comparing fresh with 6 months old (resting in -4 ºC fridge and not been used during the 6 months testing period of time) and thawed frozen DMM.

5.3.2 Results

Growth curves of the three species aerobically at 37ºC in fresh, 6 months old and thawed frozen DMM are presented in (Figures 5.5-5.7).

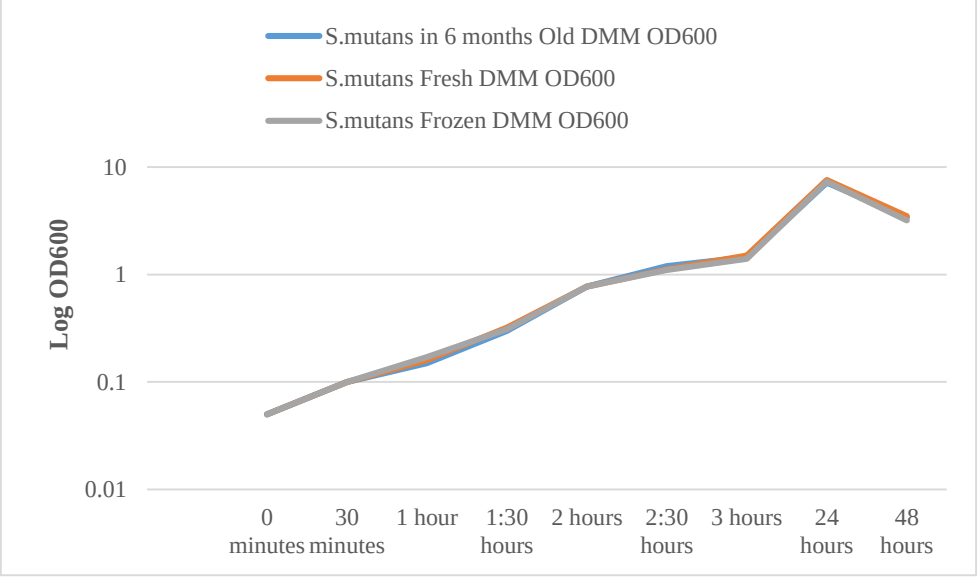


Figure 5.5 Graph represents the growth curves of *S. mutans* in fresh, 6months old and thawed frozen DMM.

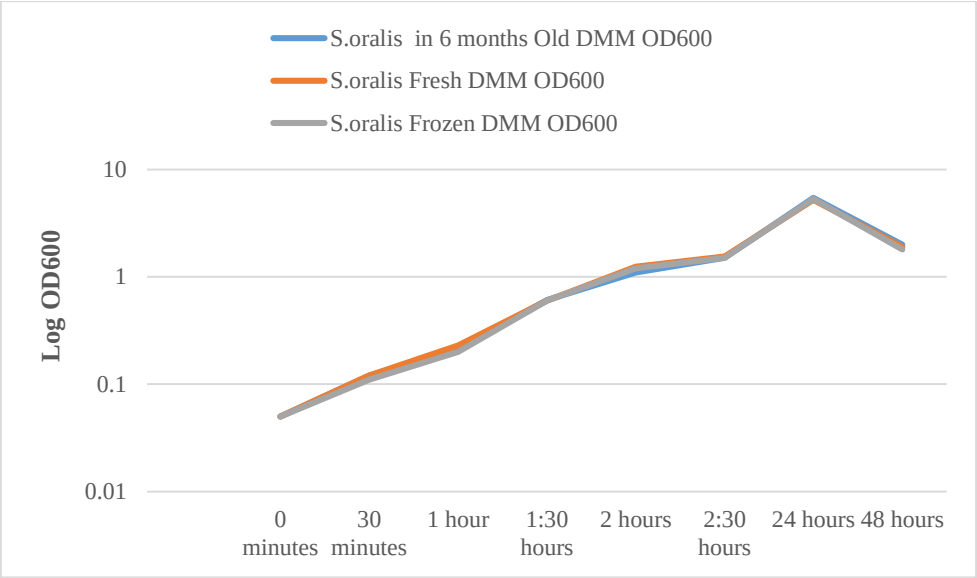


Figure 5.6 Graph represents the growth curves of *S. oralis* in fresh, 6months old and thawed frozen DMM.

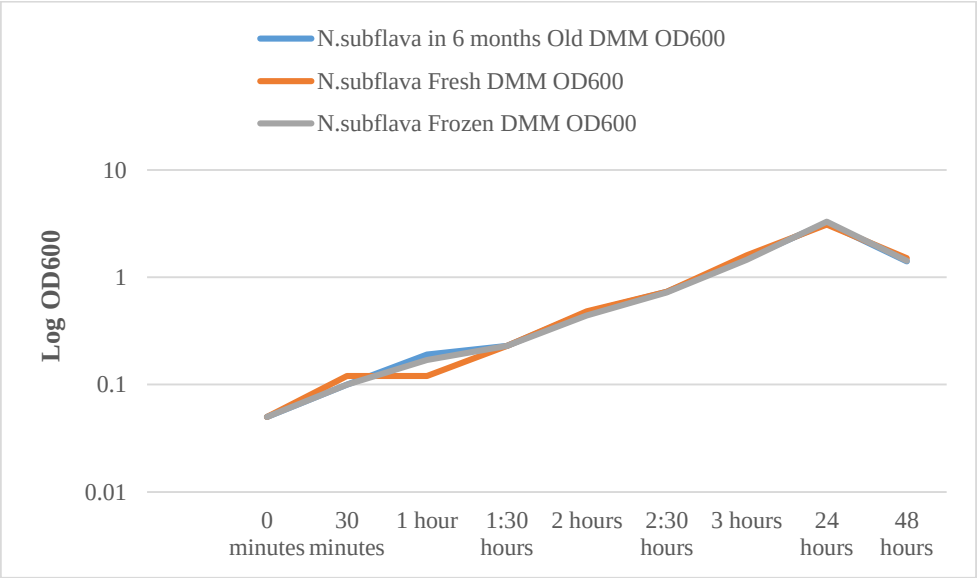


Figure 5.7 Graph represents the growth curves of *N. subflava* in fresh, 6months old and thawed frozen DMM.

5.3.3 Discussion

Natural saliva flows continuously, albeit at varying rates during any 24Hrs period, into the oral cavity, contrary to artificial saliva in *in vitro* batch biofilm models. Consequently, the provided nutrients and minerals by artificial saliva will be consumed with time unless the media is changed.

The longevity and reproducibility of the *in vitro* early coloniser model is dependent upon the availability of nutrients to the species in a steady state, so the comparison between biofilms formed in different conditions is feasible. DMM is nutritive artificial saliva contains a wide range of amino acids, growth factors and vitamins which are sensitive to temperature like asparagine, cysteine, glutamine, tryptophan, basal salivary amino acids, combined vitamin/ growth factor, urea, $CaCl_2$ and ascorbic acid (Wong and Sissons, 2001), and other components may degrade with time. This degradation may affect the biofilm formed by affecting the growth of each species either by retarding their growth or by enhancing one of them in favour of others.

In this experiment, neither DMM freezing nor 6 months aging seemed to have an apparent effect on the growth of early coloniser species aerobically as shown in Figures 5.5-5.7.

Cells were dividing at the same speed and pattern at the three conditions of DMM, *S. oralis* and *S. mutans* are facultative anaerobic Gram-positive streptococci (Figure 5.1 B and C), while *N. subflava* is a Gram-negative obligate aerobic diplococcus (Figure 5.1 A). *N. subflava* has a reasonably broad range of nutritional requirements, unlike *N. meningitides* and *N. gonnohrea* which require blood or serum in the media "Chapter 5 - Oral Mucosal Microbes" (2015) similarly, *S. mutans and S. oralis.* Therefore, the likelihood of degradation of certain vitamins and growth factors of being significant within the parameters of our assay is negligible.

5.4 The Effect of Six Well Plate on the Growth of Single Species and Mixed Species of Early Coloniser Model at Different ODs

To form multispecies biofilm, early coloniser species must grow together in a community. In a mixed community species normally compete for nutrients, in addition cell-cell interaction occur. This experiment was designed to test the growth of early coloniser species together in a vessel. In this vessel the *in vitro* model was formed over restorative material specimens.

5.4.1 Materials and Methods

5.4.1.1 Bacterial Species; *S. mutans, S. oralis* and *N. subflava.*

5.4.1.2 Growth Medium; DMM and CB**.**

5.4.1.3 Culturing Methods and Growth Conditions

An overnight culture for each species was prepared in CB aerobically, then they were diluted back to OD_{600} 0.05 in CB using the protocol in section (5.1.1.3).

Growth was monitored till mid-exponential then diluted back to OD_{600} 0.05, after that growth was monitored again till mid-exponential. Six well plate (Greiner CELLSTAR®, Sigma-Aldrich Company Ltd. Dorset, England) was chosen to host cultures. They are sterile plates with non-treated surfaces to exclude any effect on bacterial growth. In addition, they have the needed well size which will hold 5ml of media leaving sufficient air space for oxygen uptake.

Species were inoculated in wells to make final OD_{600} 0.1 with DMM, this OD was considered a dilution form mid-exponential which means cells are dividing and highly viable. Species were inoculated according to the arrangement listed in (Table 5.1) and (Figure 5.8). At this OD_{600} 0.1, the average number of inoculated species was known from pilot experiments were conducted to assess the CFUs/ml for each species at that OD_{600}

(data not shown), and because they were a dilution from mid-exponential they were doubling and highly active.

After inoculation, the six well plate was incubated in an aerobic incubator at 37°C, after 24Hrs each well content was pipetted in a labelled tube and the wells were filled with 5ml sterile PBS and left shaking for 1hour in a washing step to assay what was loosely attached to the well surfaces.

For wells 1, 2 and 3 which contained single species culture, only an OD_{600} was taken and a specimen was checked under the microscope to make sure of growth and exclude contamination. But for wells 4,5 and 6 each the planktonic growth in wells (Floating) and the PBS from washing step (loosely attached) was assayed by setting up a dilution series and measuring the CFUs using the protocol described in section (4.2.2). This experiment was repeated three times to check for a consistent result.

Table 5.1 Wells numbers and their correspondence inoculated species.

Well number	**Inoculated species**
1	*N. subflava.*
2	*S. mutans.*
3	*S. oralis.*
4	*N. subflava, S. oralis.*
5	*S. mutans, S. oralis.*
6	*S. mutans, S. oralis, and N. subflava.*

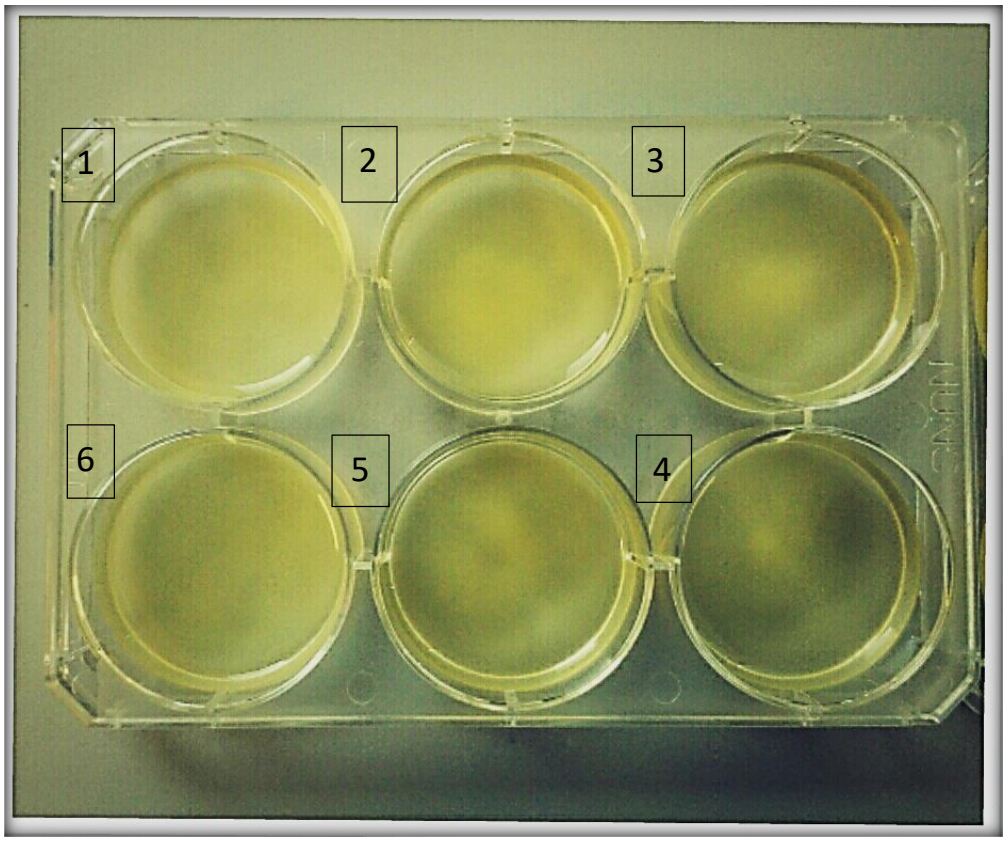


Figure 5.8 Six well plate, wells numbered from 1-6, species were inoculated in each well according to Table 5.1 in DMM to make a final 5ml volume in each well. Turbidity in the wells represent bacterial growth.

5.4.2 Results

From wells with single species OD_{600} readings were in stationary phase, the wells contained the intended species after checking light microscopic images (Data not shown).

In mixed species wells, CFUs resulted assay are presented in (Figures 5.9-5.11). For CFUs assay, two types of agar plates were used as stated before; CB plates to differentiate between total streptococci and *N. subflava*, and MSA plates in which only streptococci can grow, in addition *S. mutans* colonies gave a yellow hallow indicating mannitol fermentation (Figure 5.12).

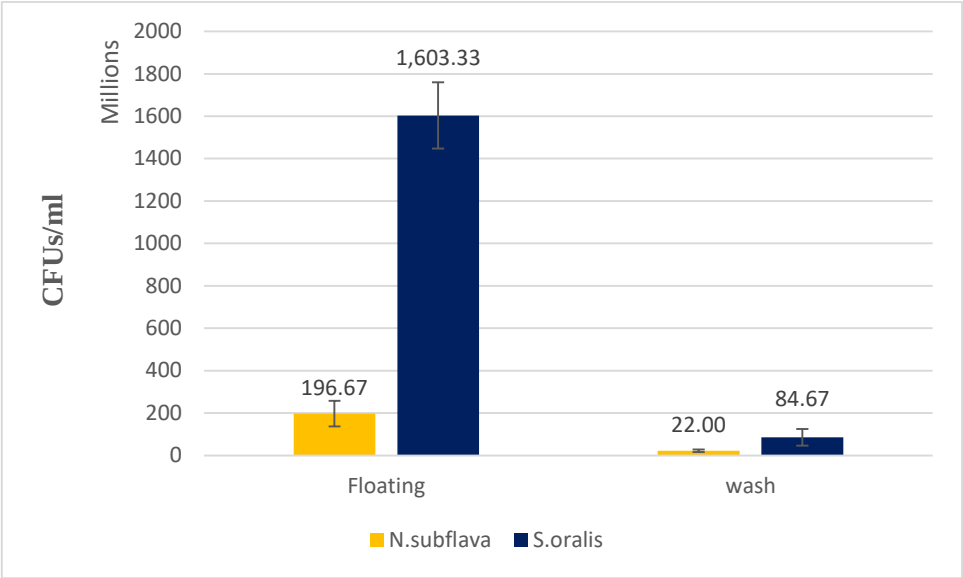


Figure 5.9 Viable cell counts assayed (CFUs) (Mean ± SEM *n* =3) from wells number 4, which contains *S. oralis* and *N. subflava* only.

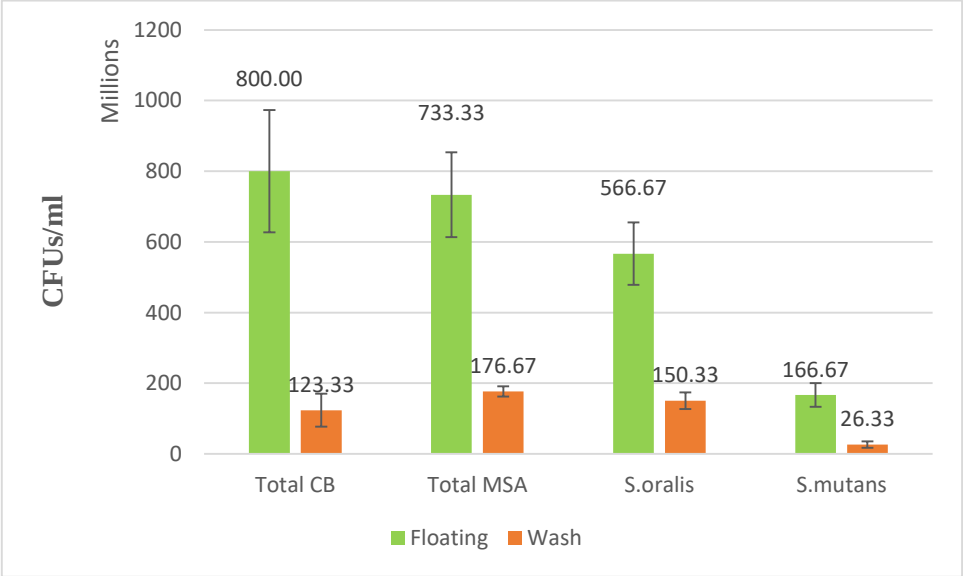
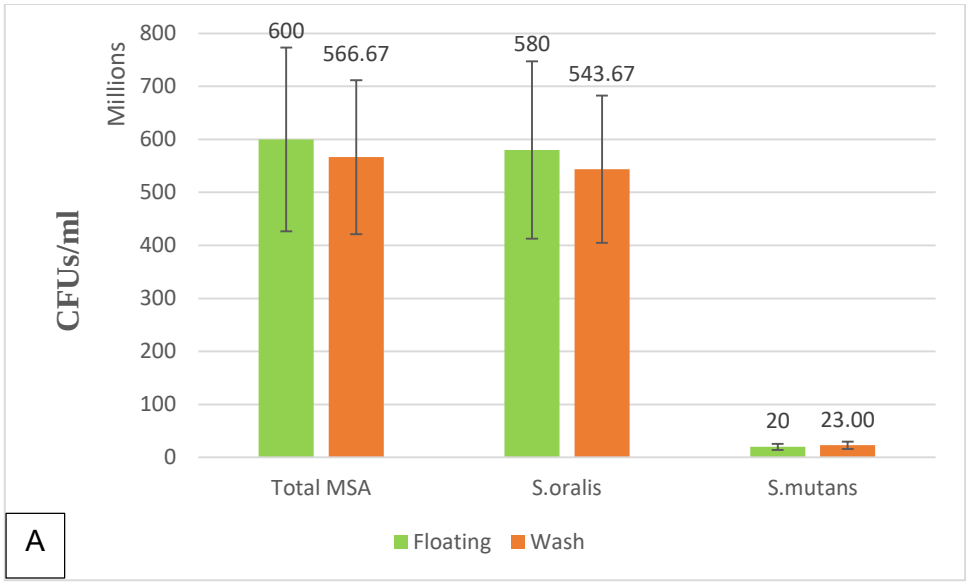


Figure 5.10 Viable cell count assayed (CFUs) (Mean ± SEM *n* =3) from wells number 5, which contains *S. mutans* and *S. oralis* only.



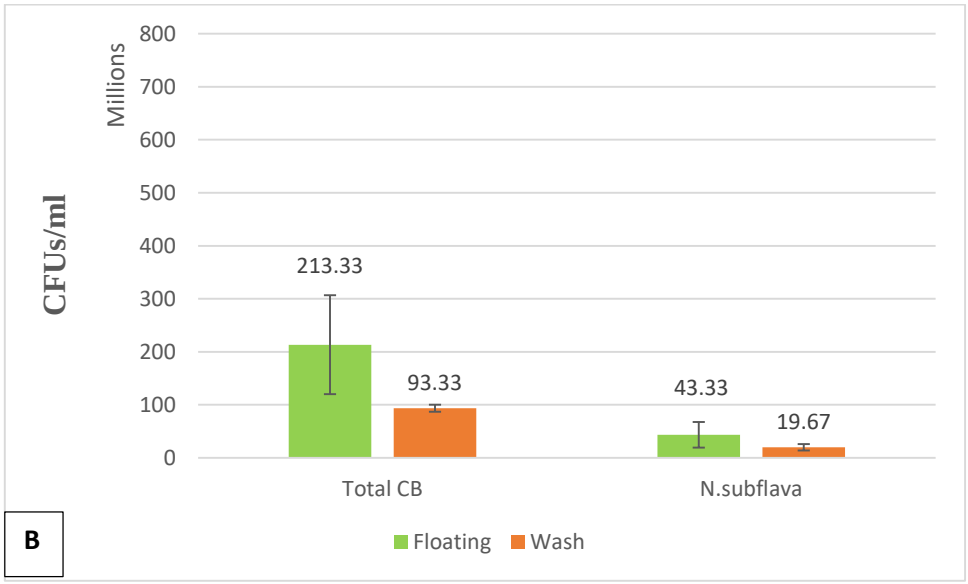


Figure 5.11 Viable cell count assayed (CFUs) (Mean ± SEM *n* =3) from wells number 6, which contained *S. mutans*, *S. oralis* and *N. subflava*. Graph A is what been assayed from MSA plates (Mannitol Salt Agar), whereas Graph B what been assayed from CB plates (Columbia Broth).

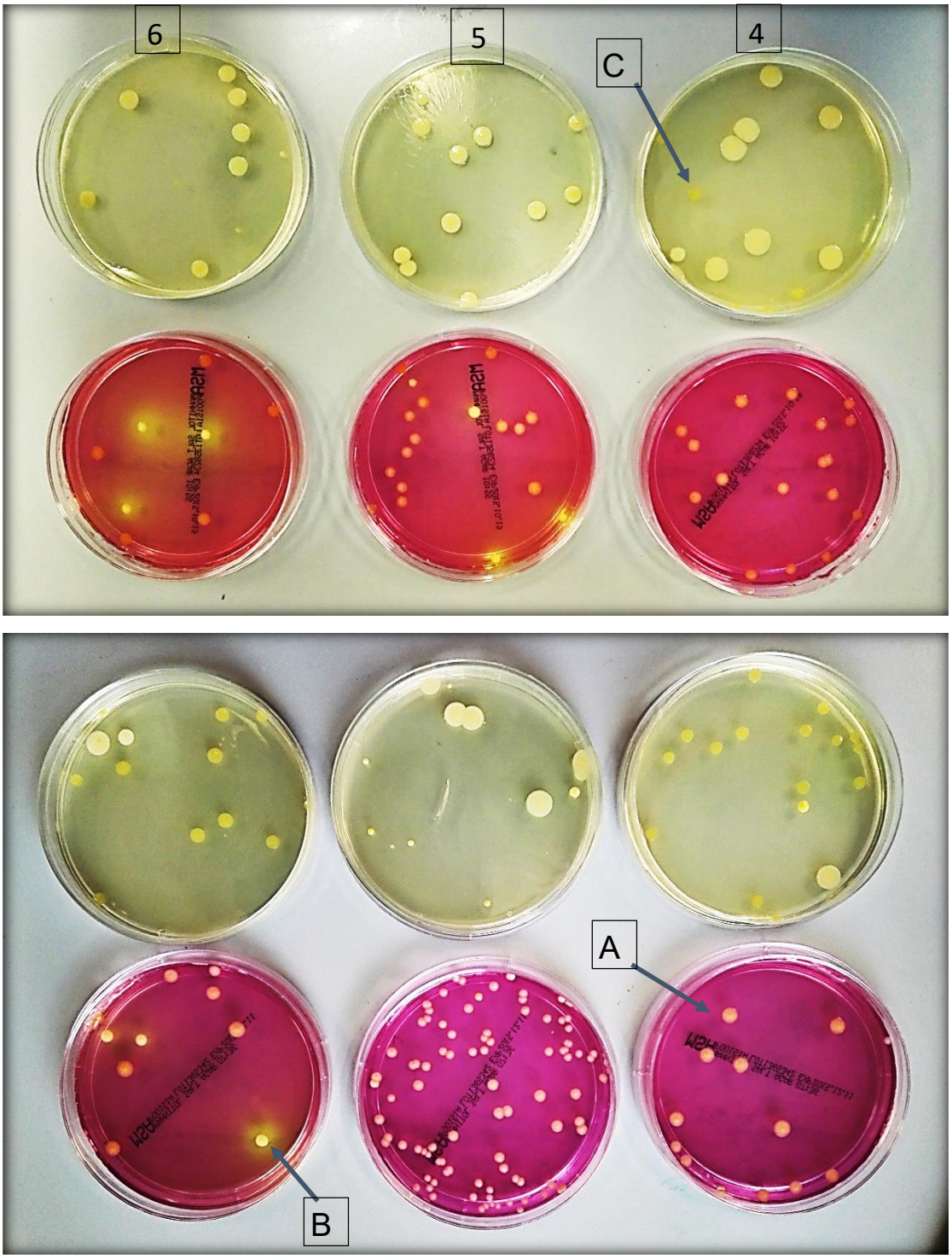


Figure 5.12 CFUs assay of Floating and Washed bacterial species by plating out on CB agar plates (pale yellow) and MSA plates (pink). For wells number 4, 5 and 6. MSA as stated earlier is selective to streptococci in general and for *S. mutans* in particular (arrow B), colony has a yellow halo indicating mannitol fermentation. On CB plates; *N. subflava* (arrow C) give different colony from streptococci. Arrow A points to *S. oralis*.

5.4.3 Discussion

The formation of biofilm needs multispecies growth in one community. From the previous experiments the conditions around early coloniser species growth in single cultures was assessed, but their growth in a different vessel and in a mixed form was tested in this experiment.

To study the behavior of those species as a mix; *S. oralis* was mixed first with *S. mutans* then with *N. subflava* in a separate well, consequently their growth could be compared with each other's and to their growth when they were all three together.

In wells with single species culture, cultures were at stationary phase reflected from OD_{600} readings and light microscope check.

CFUs assay resulted from wells with mixed species reflected their behavior. In addition, plating out using two types of agar plates resulted in a thorough and detailed output for the survival of this bacterial community in total and for each species.

Although most of the previous studies used to assay bacterial attachment on restorative material surfaces (Chen et al., 2014, Cheng et al., 2012a) ignored the effect of restorative materials on the planktonic growth around them. This might result in an incomplete understanding of the antibacterial mechanisms if any additives would be examined i.e. is it by direct contact killing, release in the system or both. Consequently, the planktonic growth in the wells was essential to assay because introducing restorative materials in this system may affect the system ecology by releasing of antimicrobials or competing for colonisation.

The second output assayed from each well was identified as Wash' which is simply the loosely attached bacteria to well surface after incubation, because it can be assumed that the presence of restorative material in the well system may compete with well surfaces

on the colonisation of biofilm species. The loosely attached community was assayed by plating out the PBS resulted from wells washing after one-hour shaking.

In wells contained *S. oralis* with *N. subflava*; *S. oralis* was a dominant growing bacteria with a mean total of 1.6×10^9 CFUs/ml, while *N. subflava* is 1.9×10^8 (Figure 5.9).

In the same wells, the loosely attached bacteria on the well surface which assayed and named as Wash CFUs was always less than the planktonic growth of bacteria in the well itself which also named as Floating CFUs.

In wells containing *S. oralis* and *S. mutans*; also *S. oralis* dominating the growth with mean total of 5.6×10^8 and mean total of *S. mutans* is 1.6×10^8. The washed assayed bacteria were always less than what's floating in the wells (Figure 5.10).

In wells containing three species, *S. oralis* was dominating the other species with total mean of 5.8×10^8 from the Floating assay and 5.4×10^8 from Wash assay (Figure 5.11 A).

When the three species are together the washed assay increased especially the CFUs retrieved from MSA plates. But on CB plates the Floating assay still more than the washed assay (Figure 5.11).

S. oralis was consistently a dominating bacteria in mixed community with either *N. subflava* or *S. mutans* or both within the parameters of this model. To some extent this result was close to natural oral biofilm formation where 80% of the early coloniser community is facultative anaerobes streptococci and *S. oralis* is the pioneer coloniser (Kolenbrander et al., 2010).

As a result of the establishment of consistent and reproducible multispecies community of the early coloniser species in six well plate, advancement toward restorative materials inclusion in the system is advisable.

5.5 Determination the Optimum UV Energy for Specimen's Sterilisation

Disinfection or sterilisation procedures of dental restorative materials has an effect on the properties of the materials which would result in a different material to what is originally tested and therefore results may not be extrapolated clinically, in other words that may affect the colonisation and biofilm formation over these materials (Farrugia et al., 2015).

Exposure to ethanol has been shown to affect physical properties of dental composites composed of both methacrylate and silorane resins (Benetti et al., 2013). Exposure of dental materials to high temperatures by immersion in hot solution containing ethanol results in higher water sorption, and increased degree of conversion and polymerization of composites (Kim et al., 2013). Gamma radiation has also been shown to lead to an increase in the resins' conversion degree, due to Gamma radiation's higher penetration power compared to that of visible light. Gamma radiation also was found to increase surface hardness and decrease water sorption and solubility (Carvalho et al., 2009).

Steam sterilisation has been claimed to cause extensive material degradation because of the high temperatures required for the process. Furthermore, steam has been shown to degrade polyurethane and affect dimensional stability of polyethylene (Zhang et al., 1996). Use of ethylene oxide has therefore been suggested for temperature, moisture and radiation sensitive products.

Ethylene oxide which is the method most used in the literature does not affect material properties but was found to be ineffective in sterilising Nano-composites; on the other hand, autoclaving appears to be a more suitable technique with no resultant degradation of material and successful sterilisation was reported (da Cunha Mendes et al., 2008).

Considering all the issues around different methods, the decision was taken to prepare materials under as sterile conditions as possible (i.e. using sterile gloves and sterile water, glass slabs were wiped with 100% ethanol), then the disks were sterilised using UV radiation because it was found to have the least affect, after ethylene oxide, on micro-hardness of ChemiFil® Superior (the restorative material am testing in this project) (Figure 5.13) (Farrugia et al., 2015).

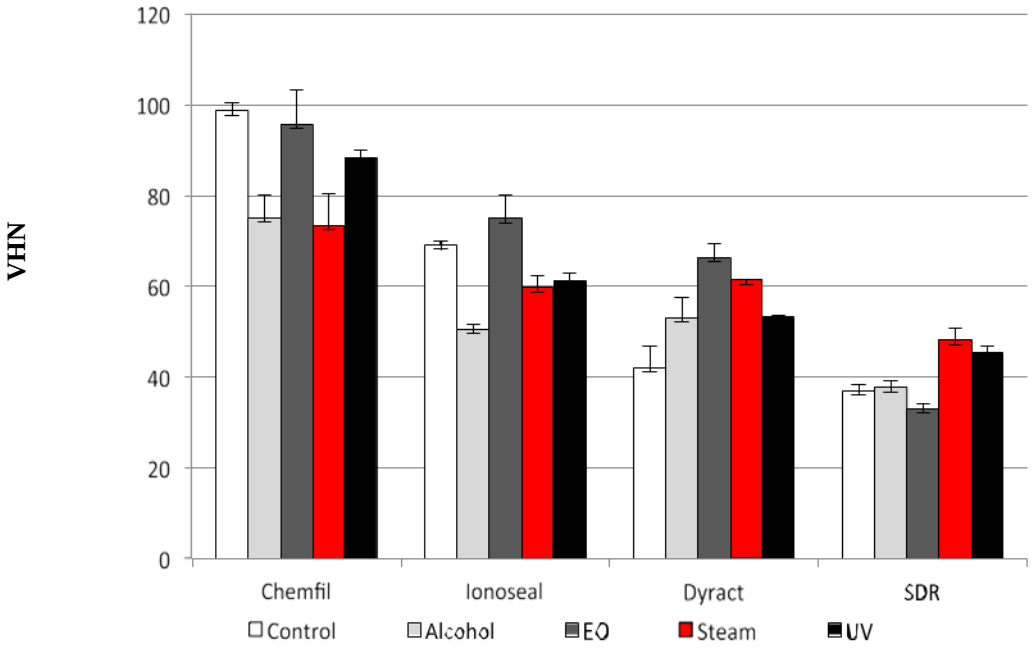


Figure 5.13 Graph showing the effect of different sterilisation methods on the micro-hardness measured using Vickers hardness number (VHN) of different restorative materials. ChemiFil® the restorative material used in this project showing that UV radiation has the second least effect on micro-hardness (Farrugia et al., 2015).

To determine the effective UV energy dose needed to decontaminate and sterilise GIC disks after preparation an experiment was designed as following.

5.5.1 Materials and Methods

18 GIC disks were prepared according to manufacturer's instructions using the criteria mentioned in section (4.1.3.1). Those 18 disks were divided into 3 groups of 6 disks, first group was subjected to 8000 µj/cm^2 while the second 16000 µj/cm^2 and the third 32000 µj/cm^2 on both sides of each disk because the source of UV radiation comes only from the top of the UV machine (UVP CL-1000 cross-linker, Cambridge UK).

After that, each set were inserted into a labelled six well plate contained 5 ml of sterile DMM in each well. Plates were incubated aerobically at 37°C incubator; in addition, one six well plate was used as control and contained only sterile DMM.

OD_{600} was checked and 100µl of each well were plated on sterile Blood agar plate every 24Hrs for consecutive three days.

5.5.2 Results

Tables 5.2-5.5 represent the optical density readings and any positive growth on blood agar plates from tested wells with GIC disks administrated the examined sonication energy.

Table 5.2 Six wells OD_{600} reading and positive growth (+) contained GIC disks after administration of 8000 µj/cm^2 UV energy.

8000 µj/cm^2		
	After 24Hrs	
Well number	**OD_{600}**	**Growth**
1	**0**	**+**
2	**0**	**+**
3	**0**	**+**
4	**0**	**+**
5	**0**	**+**
6	**0**	**+**

Table 5.3 Six wells OD_{600} reading and positive growth (+) contained GIC disks after administration of 16000 µj/cm^2 UV energy.

16000 µj/cm^2						
	After 24Hrs		**After 48Hrs**		**After 72Hrs**	
Well number	**OD_{600}**	**Growth**	**OD_{600}**	**Growth**	**OD_{600}**	**Growth**
1	**0**	**-**	**0**	**+**	**-----**	**+**
2	**0**	**-**	**0**	**+**	**-----**	**+**
3	**0**	**+**	**0.2**	**+**	**-----**	**+**
4	**0**	**-**	**0.1**	**+**	**-----**	**+**
5	**0**	**-**	**0**	**-**	**0.3**	**+**
6	**0**	**-**	**0**	**-**	**0.2**	**+**

Table 5.4 Six wells OD_{600} reading and positive growth (+) contained GIC disks after administration of 32000 µj/cm^2 UV energy.

32000 µj/cm^2						
	After 24Hrs		After 48Hrs		After 72Hrs	
Well number	OD_{600}	Growth	OD_{600}	Growth	OD_{600}	Growth
1	0	-	0	-	0	-
2	0	-	0	-	0	-
3	0	-	0	-	0	-
4	0	-	0	-	0	+
5	0	-	0	-	0	-
6	0	-	0	-	0.1	+

Table 5.5 Six wells OD_{600} reading and positive growth (+) contained only sterile DMM without any UV energy.

Control (DMM only)						
	After 24Hrs		After 48Hrs		After 72Hrs	
Well number	OD_{600}	Growth	OD_{600}	Growth	OD_{600}	Growth
1	0	-	0	-	0	-
2	0	-	0	-	0	-
3	0	-	0	-	0	-
4	0	-	0	-	0	-
5	0	-	0	-	0	-
6	0	-	0	-	0	+

5.5.3 Discussion

The administration of UV radiation at 8000 µj/cm^2 to both top & bottom surfaces of the disk resulted in OD_{600} of zero after 24Hrs incubation. However, upon plating out 100 µls onto Blood Agar there was a growth in all wells & it was decided a higher does was required (Table 5.2).

Administering 16000 µj/cm^2 a growth in one well resulted after 24Hrs with OD_{600} equals to zero, however in 2 wells changing in OD_{600} was noticed after 48Hrs and growth detected on Blood agar plates from all wells by day 3 (Table 5.3).

Doubling the amount of UV irradiation to 32000 µj/cm^2 didn't show any growth after the first and second day in all wells; after the third day only two wells revealed growth, one of them associated with zero OD_{600} (Table 5.4).

DMM showing a good sterility through 72Hrs incubation, one well showed growth with zero OD_{600} after 72Hrs suggesting pipetting contaminant (Table 5.5).

32000 µj/cm^2 is the maximum energy power provided by the UV cross-linker, the time could be increased but within our biofilm assay parameters this energy is adequate because we are concerned only with early colonisation within the first 24Hrs and 48Hrs and aiming to minimise any exposure to the restorative material as possible. Occasional contamination may occur at any time within the experiments and it could be discovered by checking microscopically or after plating on agar plates.

So, UV irradiation proved to be a suitable non-invasive method for sterilising Glass ionomer cements.

5.6 Comparing Three Times Washing Series and Five Times Washing Series

Batch *in vitro* models are closed systems, the environment inside the wells changes with time during the test as nutrients are consumed and metabolic products accumulate unless the growth media are replaced (Coenye and Nelis, 2010). The frequency for changing the growth media depends on the model set up.

The lack of fluid flow and continuous nutrient provision to the system is one of Batch culture drawbacks. Although some models have been adapted to create a liquid shear force by dipping the biofilms in saline or other liquid during biofilm formation (Guggenheim et al., 2004), there wasn't any consistent method in adapting shear force to batch cultures. Artificial mouth is superior to Batch models in that case, however batch models still represent relatively simple environments that can be reproduced with minimal set up time (Coenye and Nelis, 2010).

Doing a serial wash of the resultant disks holding the biofilm was the decision in a way to overcome that drawback by mimicking the saliva shear force and remove the loosely attached bacteria which might overestimate the results.

Several large studies have reported that the mean flow rate of unstimulated/resting whole saliva in healthy persons during the day is in the range of 0.3–0.4 ml/min but with a large standard deviation (Heintze et al., 1983).

Salivary flow rate shows a circadian rhythm of high amplitude with peak flow in the late afternoon, while the flow rate is extremely low during sleep, which reduces the need to swallow during that time (Dawes, 1974). The total volume of saliva secreted per day has been estimated to be about 0.6 L (Heintze et al., 1983).

Washing time was fixed to one hour in two suggested methods, the first was 15ml of sterile PBS on three serial washes each time 5ml for 20 minutes, the second was 25ml of sterile PBS on five serial washes each time was 5ml for 12 minutes.

The following experiment was to determine the efficiency of those two methods in removing the unwanted friable bacteria from biofilm surface.

5.6.1 Materials and Methods

5.6.1.1 Bacterial Species; *S. mutans, S. oralis* and *N. subflava.*

5.6.1.2 Growth Medium; DMM and CB.

5.6.1.3 Culturing Methods and Growth Conditions

Each species was grown by the same method in section (5.2.1.3) to have a mid-exponential cultures then in a sterile six well plate, wells were inoculated with each species at a final OD_{600} 0.1 and then the wells were topped up with sterile DMM to a final volume of 5ml.

12 GIC disks were prepared according to the manufacturer's instructions using the protocol described in section (4.1.3.1) and then they were sterilised by UV radiation at 32000 μj/cm^2 on both sides. The sterile disks were inserted in the inoculated wells and then incubated in an aerobic cabinet for 24 hours at 37°C. 6 discs from first well plate were transferred gently to a new six well plate containing 5ml sterile PBS in each well. The plate was left shaking for 20 minutes, then the disks transferred to a new six well plate for another washing step till three washes. Six disks from the second well plate were washed in the same way as the first plate except they were washed for 5 times each time was 12 minutes.

For each time the disks transferred to a fresh plate, the PBS around the disks after shaking was assayed for CFUs by making a dilution series. Mean CFUs from each washing step was plotted to compare both washing series as shown in the graphs below.

5.6.2 Results

The reduction in total bacterial CFUs from each wash of both washing series is presented in Figures 5.14 and 5.15.

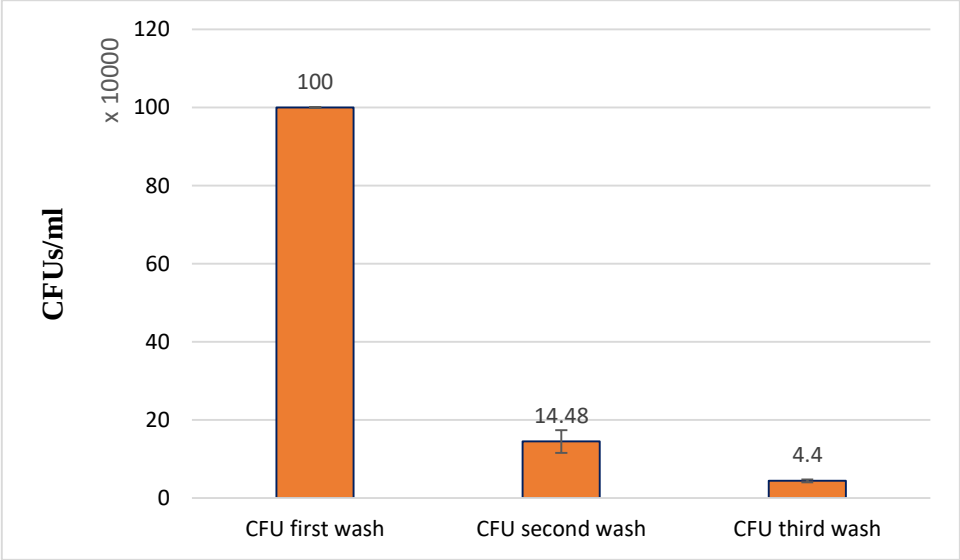


Figure 5.14 Graph for the viable cell counts CFUs (Mean ± SEM *n* =6) assayed from GIC disks holding 3D biofilm from each wash of three washing series each on for 20 minutes.

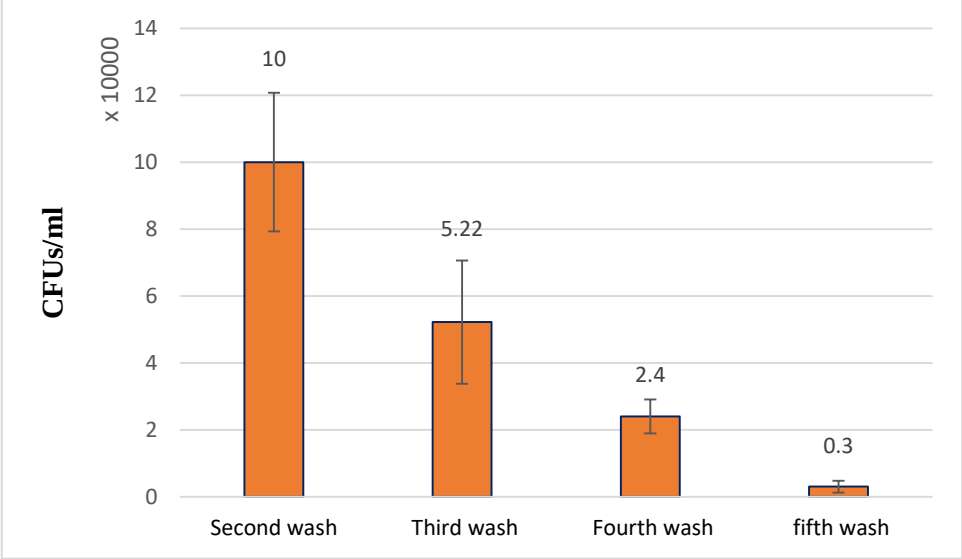


Figure 5.15 Graph for the viable cell counts assayed CFUs (Mean ± SEM *n* =6) from each washing step (12minutes) of five to GIC disks holding 3D biofilm, first wash assay was over 1×10^6 CFUs/ml and omitted from the graph to clarify the reduction from second to fifth wash.

3.6.1 Discussion

From (Figure 5.14), three steps washing successfully decreased the mean total number of loosely attached bacteria in each step starting from more than 1×10^6 CFUs /ml to 1.4×10^5 in the second step and 4×10^4 in the last wash. Whereas with five washing steps (Figure 5.15), the reduction in the total number of loosely attached bacteria starting from the first step which was more than 1×10^6 CFUs/ml (it was deleted from the chart to clarify the trend of reduction till the fifth step), the second 1×10^5, the third 5.22×10^4, the fourth 2.41×10^4 and the fifth was 3×10^3.

The reduction percentages in three washes were 14%, then 31%. But reduction percentages of five washes were 10%, 52.2%, 45.9% and last one was 12.5%. The slope of reduction with five times washing series was clearer compared to three washes.

From both methods, the first wash CFUs in average was more than 1×10^6 CFUs /ml. However, the whole bacterial CFUs reduced by both methods was almost the same in average; three washing series removed around 18.88 $\times10^6$ CFUs/ml in total while five washing series removed 17.92×10^6 CFUs /ml in Total. That led to an assumption that this was the maximum number of CFUs which could be liberated within an hour of washing. Finally, both methods are efficient in removing friable bacteria from biofilms surfaces, but five washing steps gave a smoother reduction. This structured manner in washing may allow a more controlled comparison between tested samples, by eliminating the effect of friable bacteria on the end results. In addition, in this washing method all samples were treated with the same conditions which reduce the effect of subjectivity and human errors.

5.7 The Effect of Sonication on the Viability of Early Coloniser Species as a Single or Consortium

In the past decade, sonication i.e. ultrasonic energy applied directly to the biomaterial surface to disrupt adherent biofilm has been reported to be a more reliable tool for the diagnosis of Prosthetic joint infection (Tunney et al. 1999, Nguyenet al. 2002, Trampuz et al. 2007, Estebanet al. 2008). Recently in dental research, sonication and vortexing has been used to disrupt *in vitro* biofilms from tested surfaces (Cheng et al., 2012a, Cheng et al., 2012d, Li et al., 2009, Kobayashi et al., 2009).

Sonication and vortexing was selected as a method to detach the biofilm from GIC disks after incubation. However, Sonication power may affect the viability of bacterial cells by inactivation of various microorganisms (Gao et al., 2014), The microbial inactivation effect of ultrasound has been known since the early 30's (Harvey and Loomis, 1929). Since then, several investigations have been carried out to study the inactivation effect of ultrasound.

To test the effect of sonication power on the viability of early coloniser model species, the following experiment was designed.

5.7.1 Materials and Methods

5.7.1.1 Bacterial Species; *S. mutans, S. oralis* and *N. subflava.*

5.7.1.2 Growth Medium: CB.

5.7.1.3 Culturing Methods and Growth Conditions.

Each species was grown following the same method described in section (5.2.1.3), to obtain single culture at an OD_{600} of 0.1 after diluting out from mid-exponential. Then in sterile 1.5ml Eppendorf tubes, 1 ml of each single species culture at an OD_{600} of 0.1 was made in duplicates plus two of 1ml mixed culture at a final OD_{600} of 0.1.

One set of the Eppendorf tubes was inserted in a water bath of a sonicator (1510E-MT, Branson Ultrasonics, Danbury, CT) for 5 minutes at a frequency of 40 kHz, at the same time the other set was inserted in a water bath of the same volume and temperature of the sonicator water bath (room temperature).

After 5 minutes the viability of each species in a single culture and as a mix was assayed by CFUs. This experiment was repeated three times.

5.7.2 Results

The effect of sonication power is represented by change in the CFUs assayed from tested samples as a single and mixed culture community in Figures 5.16 and 5.17.

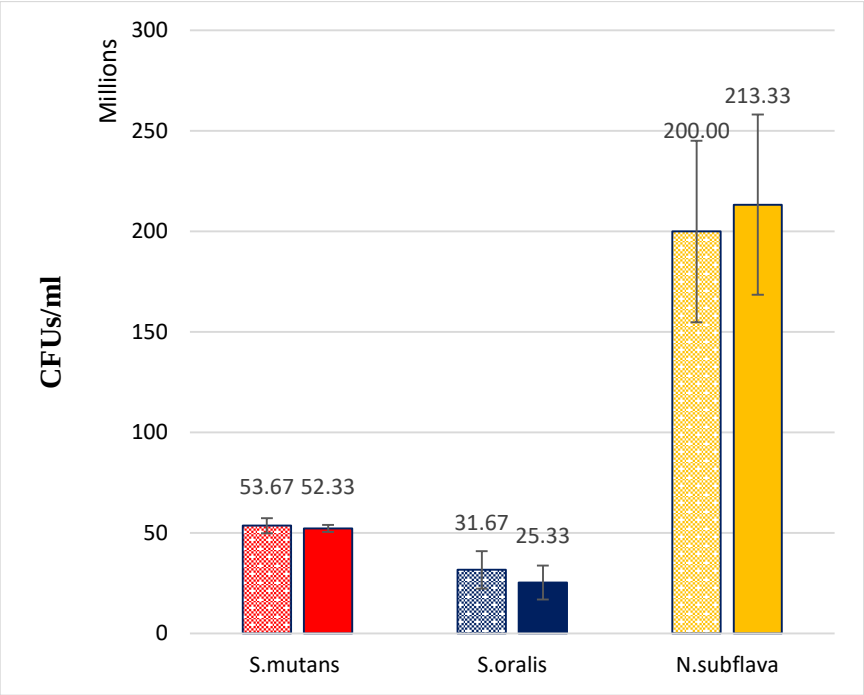


Figure 5.16 The effect of 5 minutes 40 kHz sonication power on viability of 0.1 OD_{600} single species cultures represented by the change in total CFUs (Mean ± SEM *n* =3) (Solid colors represent CFUs with sonication while patterned colors represent CFUs without sonication).

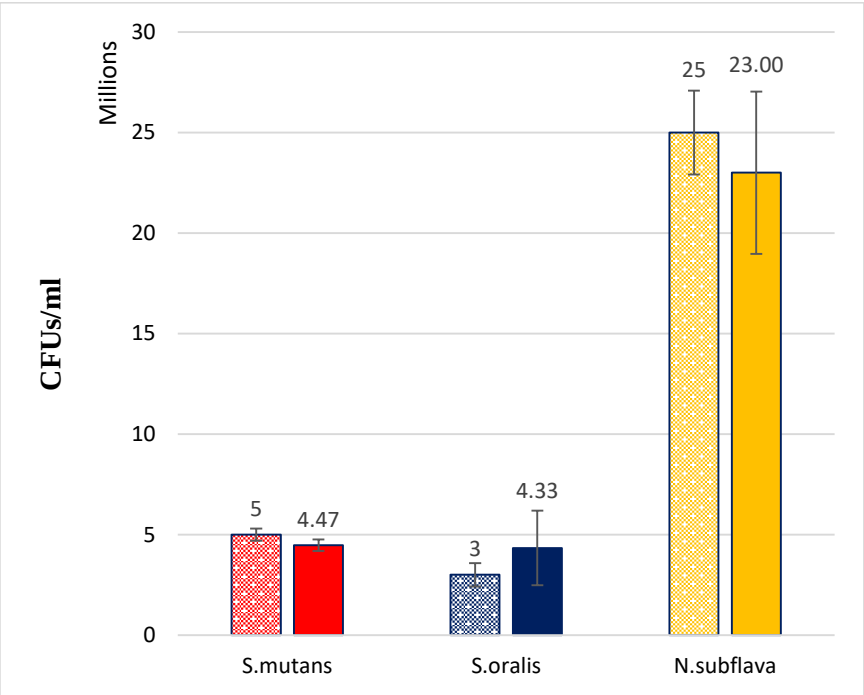


Figure 5.17 The effect of 5 minutes 40 kHz sonication power on viability of 0.1 OD_{600} Mixed species cultures represented by the change in total CFUs assay (Mean ± SEM *n* =3) (Solid colors represents CFUs with sonication while patterned colors represent CFUs without sonication).

5.7.3 Discussion

Ultrasound is defined as sonic waves with frequencies over the threshold of human hearing (16-20 kHz). Ultrasound generators generate these high frequency waves based on the properties of some piezoelectric materials. These vibrate with a specific amplitude at the ultrasonic frequency when voltages are applied as high frequency pulses (Kuo et al., 2008). High intensity ultrasound (<100 kHz) has been suggested for different applications including microbial and enzymatic inactivation from foods. When these high intensity ultrasound propagates in liquid media, the cavitation phenomena are generated. Cavitation consists on the formation, growth and suddenly implosion of bubbles. Due to the implosion, the molecules around the bubble hit each other violently creating spots of very high temperature (5500 ºC) and peaks of pressure (104-105 kPa) (L, 1989).

Microbial inactivation by ultrasound depends on several factors like microbial characteristics and environmental factors, it is admitted generally that cells of a bigger size are more sensitive to ultrasound (Ahmed and Russell, 1975) and rod shaped bacteria more than cocci (Jacobs, 1954). Gram-positive are more resistant than Gram-negative, aerobic more than anaerobic species (Ahmed and Russell, 1975), growth phase may have an effect but still not clear because some authors have found out a non-influence of this factor on the inactivation effect of ultrasound (Hamre, 1949), others have observed that cells of *E. coli* at the exponential growth phase were more sensitive to ultrasonic treatments than that at the stationary phase (Allison et al., 1996). Acidity may also play a role; it is common that microbial heat resistance decreases in acidic media. However, different authors have observed that the ultrasonic resistance of microorganisms was independent of the acidity conditions of the medium (Kinsole, 1954, Pagán, 1999). The vessel holding the bacterial culture is one of the environmental factors that may reduce the inhibition effect.

In this experiment, there was no significant reduction in bacterial viability assayed by CFUs from the three single cultures when they were inserted in the sonication bath for 5 minutes at 40 kHz frequency (Figure 5.16). That may be explained by having different environmental conditions. In addition, the vessel holding the cultures may have an adverse effect on sonication energy delivered to cultures, or may be the time was not sufficient to have killing effect. Time of exposure and wave frequency plays a major role in killing, Joce et al 2003 (Joyce et al., 2003) found a significant increase in percent kill for *Bacillus* species with increasing duration of exposure and intensity of ultrasound in the low-kilohertz range (20 and 38 kHz) the duration was 2Hrs but in this project it was 5minutes.

Despite having the same OD_{600} reading, the three species had different average CFUs i.e. for *S. mutans* and *S. oralis* it was 5.3×10^7, 3.1×10^7 CFUs/ml respectively, while *N. subflava* was 2×10^8 CFUs/ml. This could be explained by the different behavior of these species in broth cultures; streptococci can form chains and clumps (Figure 5.23) in contrary to *N. subflava*. Assuming that ultrasonic power will disrupt clumps and chains this explanation may not be the only reason. Second explanation is the size of bacterial cells, *N. subflava* is relatively smaller than *S. mutans* and *S. oralis*. However, both streptococci have relatively the same size (Figure 5.1).

Sonication power of 40 kHz for 5 minutes has no significant effect on reduction of early coloniser model species when they are in a mixed culture (Figure 5.17).

Within the parameter of this model, 5 minutes 40 kHz sonication power from sonication bath has no significant effect on the viability of the early coloniser model species either in single or mixed cultures at an OD_{600} of 0.1 diluted from mid-exponential which was supposed to be more sensitive to sonication than stationary cultures. As a result, it can be

assumed that this power will not have a significant effect on the viability of biofilm species after 24Hrs incubation when it will be used to disrupt them from restorative materials surfaces.

5.8 The Bacteriostatic Effect of Fluoride Released from GIC on the Attachment and Growth of Early Colonisers

The rate and pattern of fluoride ions release from restorative materials depends on various factors such as temperature, pH, mixing technique, powder liquid ratio, media surrounding and area that is exposed to the oral environment (Wiegand et al., 2007).

Most studies estimated fluoride release in neutral pH or inert solutions like deionised or distilled water, few studies measure the release in low pH or artificial saliva (Karantakis et al., 2000). Fluoride release at 37°C in artificial saliva during 24 hours was 1.283± 0.021 PPM[1] which approximately equals to 1.283µg/ml (Madhtastha, 2013).

However, the amount of fluoride released from each disk in 5 ml DMM may be estimated to be equal to 5.1 µg according to the formula (Fluoride release = F µg/ml × volume of solution/Area of sample in cm^2) (Karantakis et al., 2000), assuming the surface area of each disk (1×8mm) was equals to1.257 cm^2.

Total amount of fluoride in each disk could be estimated too, in this project GIC material used is ChemFil® Superior which is a fast-setting glass-ionomer restorative material consisting of a blend of alumino silicate glass and polyacrylic acid.

From the fact sheet provided by the manufacturer, each 1g of ChemFil Superior powder contains:

1- 0.84 g of Aluminium-sodium-calcium-fluoro-phosphoro-silicate in the ratios (18:9:8:16:3:46), 16% of it is fluoride.
2- 0.15 g Polyacrylic acid (MW 30000-45000).

So in each 1 g of powder there is 0.1344 g of fluoride.

For each disk, approximately 0.24 g needed which contains 0.2016 g of Aluminium-sodium-calcium-fluoro-phosphoro-silicate material.

16% of 0.2016 equals to 32.256 µg fluoride is the approximate maximum amount in each disk.

To address the bacteriostatic effect of total amount of fluoride amount in each GIC disk sample this experiment was designed.

5.8.1 Materials and Methods

5.8.1.1 Bacterial Species; *S. mutans, S. oralis* and *N. subflava.*

5.8.1.2 Growth Medium; CB, DMM**.**

5.8.1.3 Culturing methods and growth conditions

First, a mid-exponential culture for each species was obtained using the method described in section (5.2.1.3). Then in two sterile six well plates, wells were inoculated with the three species at an OD_{600} 0.1 in sterile DMM, two wells containing sterile GIC disks sterilised with UV radiation, 2 wells containing NaF at a final concentration of 6.46µg/ml (represent the total fluoride amount in each disk) the other two wells contained only three species without any additives considered to be negative control.

One plate was incubated aerobically while the other one anaerobically for 24hrs, then CFUs of the planktonic bacterial culture in all wells were assayed and the empty wells were washed for an hour with 5ml of sterile PBS by left shaking on a rocker, and the washed solution was assayed by CFUs too. This experiment was done three times and the result as follows.

3.8.1.2 Statistical Analysis.

For statistical analysis Kruskal-Wallis one-way ANOVA test was conducted to evaluate differences between the groups.

5.8.2 Results

The planktonic growth in each well (Floating) and the washed BPS (Wash) from wells surfaces presented by CFUs in Figure (5.18).

Statistical analysis details are in appendix 3 section (10.3.1).

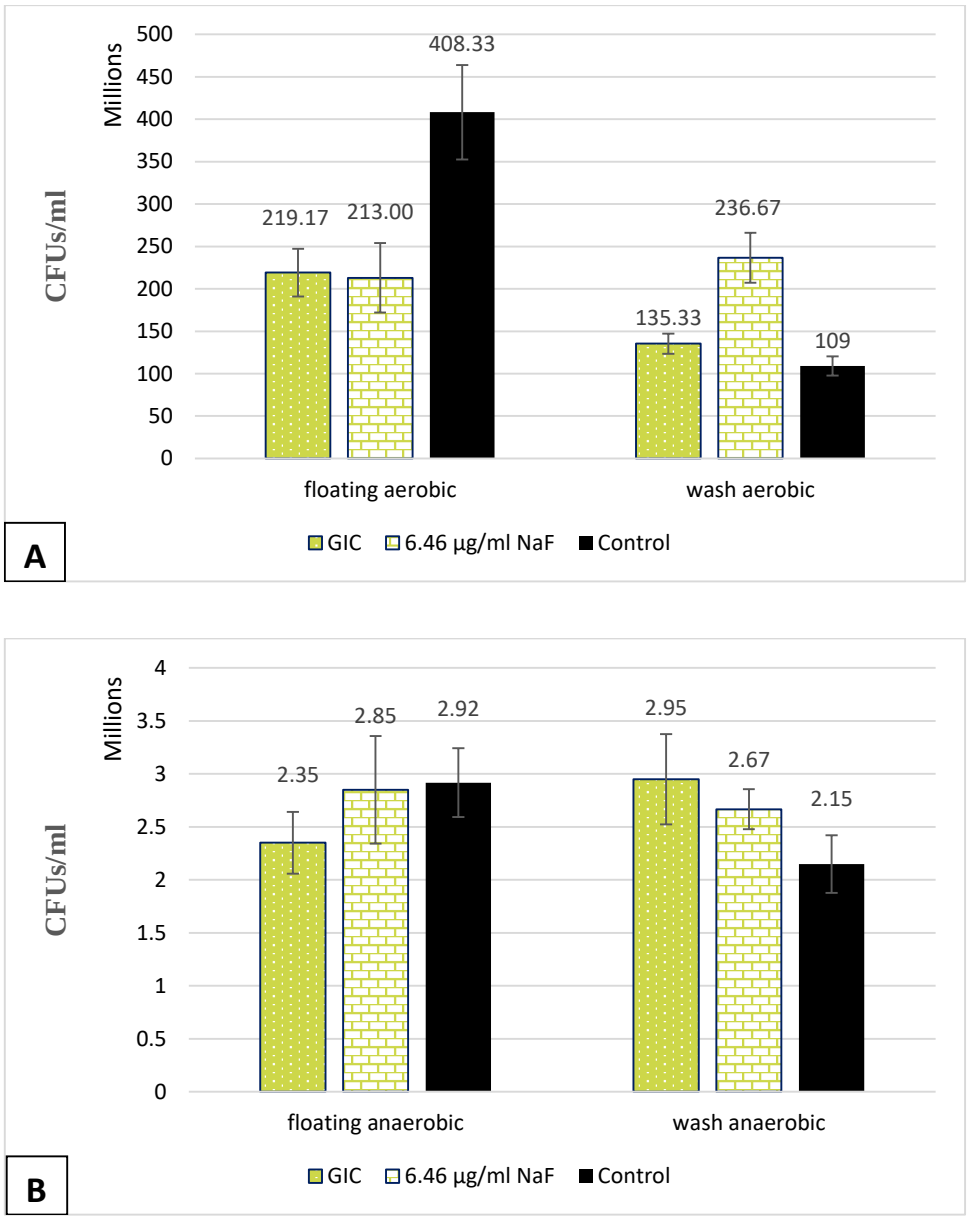


Figure 5.18 Two graphs represent the viability of early coloniser model species CFUs (Mean ± SEM *n* =6) in the presence of fluoride either released from GIC disks or as NaF solution. (6.46µg/ml represent the total fluoride present in GIC disks in form of NaF), assayed with total CFUs from the planktonic growth in the wells (Floating) and what is loosely attached to the wells surfaces (Wash) under both aerobic conditions (A), anaerobic conditions (B), Floating and Wash CFUs were significantly lower in wells containing GIC than control wells $p < 0.05$ only under aerobic conditions (Kruskal-Wallis test).

5.8.3 Discussion

Fluoride interference mechanisms with bacterial metabolism and dental plaque formation includes the inhibition of the glycolytic enzyme enolase and the proton-extruding ATPase which will affect bacterial colonisation. Furthermore, intracellular or plaque associated enzymes such as acid phosphatase, pyrophosphatase, peroxidase and catalase may be affected by fluoride ions (Hamilton, 1990).

Conflicting data resulted from *in vitro* studies on fluoride effect, which is related to experimental design differences between studies so it is difficult to compare results between studies. Most studies they used either simple tests (Agar diffusion test, MIC/MBC) or simple *in vitro* monocultures (one bacterial species). Some of these showed that low fluoride levels were released from GIC which reduced *S. mutans* growth and acid production (Friedl et al., 1997, Kan et al., 1997, Seppa et al., 1993, Seppa et al., 1995). However, in this study three microorganisms were involved as consortium to form a biofilm. From (Figure 5.18 A), in total there was a significant reduction in planktonic bacterial growth of the three species after 24hours under aerobic conditions in wells that contained GIC and 6.46µg/ml NaF compared to the control wells, while under the anaerobic conditions there wasn't any significant reduction (Figure 5.18 B). As a result of anaerobic fermentation, the acidity was assumed to be increased in cultures and fluoride either from NaF or released from the GIC worked on decreasing the acidity of the environment rather than inhibition of growth. In fact this is the second mechanism of fluoride action where it reduce the acidity and decreases the de-mineralisation of dental tissues (Nakajo et al., 2009) (Mayanagi et al., 2014). Fluoride concentrations needed for antimicrobial effects mostly surpass the concentration needed to reduce the solubility of apatite, only small amounts of fluoride (approximately 0.03–0.08 ppm) in re-mineralizing solutions are necessary to shift the equilibrium from de-mineralisation to re-

mineralisation. Therefore, it is often considered that the antimicrobial effect of fluoride is less important when the fluoride concentration required for inhibition of de-mineralisation is exceeded (Wiegand et al., 2007).

Interestingly under aerobic conditions what was loosely attached to the wells surfaces in wells with NaF was significantly higher than the other wells, in another words, the presence of NaF enhanced bacterial attachment to the well surface was more than the control and GIC disks containing wells (Figure 5.18 A). However, anaerobically although there was an increase in bacterial attachment to well surfaces containing either GIC or NaF compared to control but it was not significant (Figure 5.18 B).

The effect of total fluoride present in GIC disks on growth inhibition was comparable to the effect of fluoride released from GIC disks aerobically, which gives an indication of that the fluoride presence in GIC has an antibacterial activity and is potent to inhibit the growth of these species aerobically but not anaerobically.

This experiment was conducted to rule out the effect of fluoride presence and release from GIC at base line before adding silver, those results helped in modelling other experiments in this study, further information and results will be in the next experimental chapter.

In conclusion, Fluoride presence in GIC seems to have a bacteriostatic effect on the early coloniser model species, but it was limited to aerobic conditions only; indicating the debatable effect of fluoride. Using multispecies batch model is a useful method to detect the effect of antibacterial components of restorative materials, in which results are consistent and could be compared under different conditions.

5.9 The Attachment and Survival of Early Coloniser Model Species on GIC Surface after 24Hrs under Aerobic Conditions

In the previous experiments, emphasis was on the effect of GIC components on the growth of early coloniser species but not on their attachment and adhesion to GIC surface. In this experiment the attachment and survival of early coloniser model species was studied in further details as a base line before addition of silver. SEM was used to validate five times washing series and sonication methods.

5.9.1 Materials sand Methods

5.9.1.1 Bacterial Species; *S. mutans, S. oralis* and *N. subflava.*

5.9.1.2 Growth Medium; DMM and CB.

5.9.1.3 Culturing Methods and Growth Conditions

Each species was grown by the same method in section (5.2.1.3), to obtain single culture at an OD_{600} of 0.1 after diluting out from mid-exponential then in a sterile six well plate, wells were inoculated with each species at a final OD_{600} 0.1 and then wells topped up with sterile DMM to a final volume of 5ml. 12 GIC disks were prepared according to manufacturer instructions then they were sterilised by UV radiation at 32000 µj/cm^2 both sides.

Sterile disks were inserted in the inoculated wells and then incubated in aerobic cabinet at 37ºC for 24Hrs. After incubation, nine disks carrying 3D biofilm were washed using five times washing series, three of the washed plus the remaining non washed three disks then were fixed and prepared for SEM using protocol in section (4.5.2).

The remaining six washed disks were inserted in Eppendorf tube with 1ml of sterile PBS and sonicated in sonication bath for 5 minutes at 40 kHz, then each tube vortexed for 1 minute at maximum speed. The resulting detached bacteria from each disk which present

in each tube were assayed with CFUs and named (Intimately attached) CFUs. Three disks out of the six disks which were sonicated and vortexed were fixed and prepared for SEM using the protocol described in section (4.5.2) to validate complete detachment.

5.9.2 Results

In this experiment only the attached species to GIC were assayed, their CFUs are presented in (Figure 5.19).

SEM images for early coloniser model biofilm without washing series, after five times washing and after sonication and vortexing are presented in Figures 5.20-5.22.

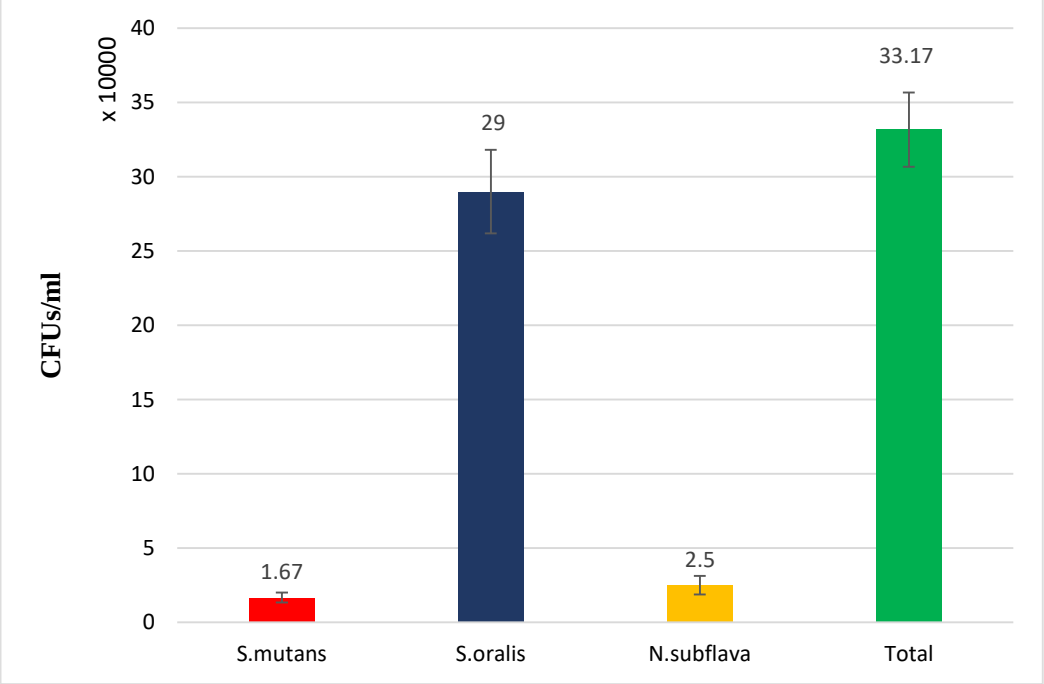


Figure 5.19 Viable cell count assay CFUs (Mean ± SEM *n* =6) of bacterial cells detached from GIC disks with sonication and vortexing (Intimately attached).

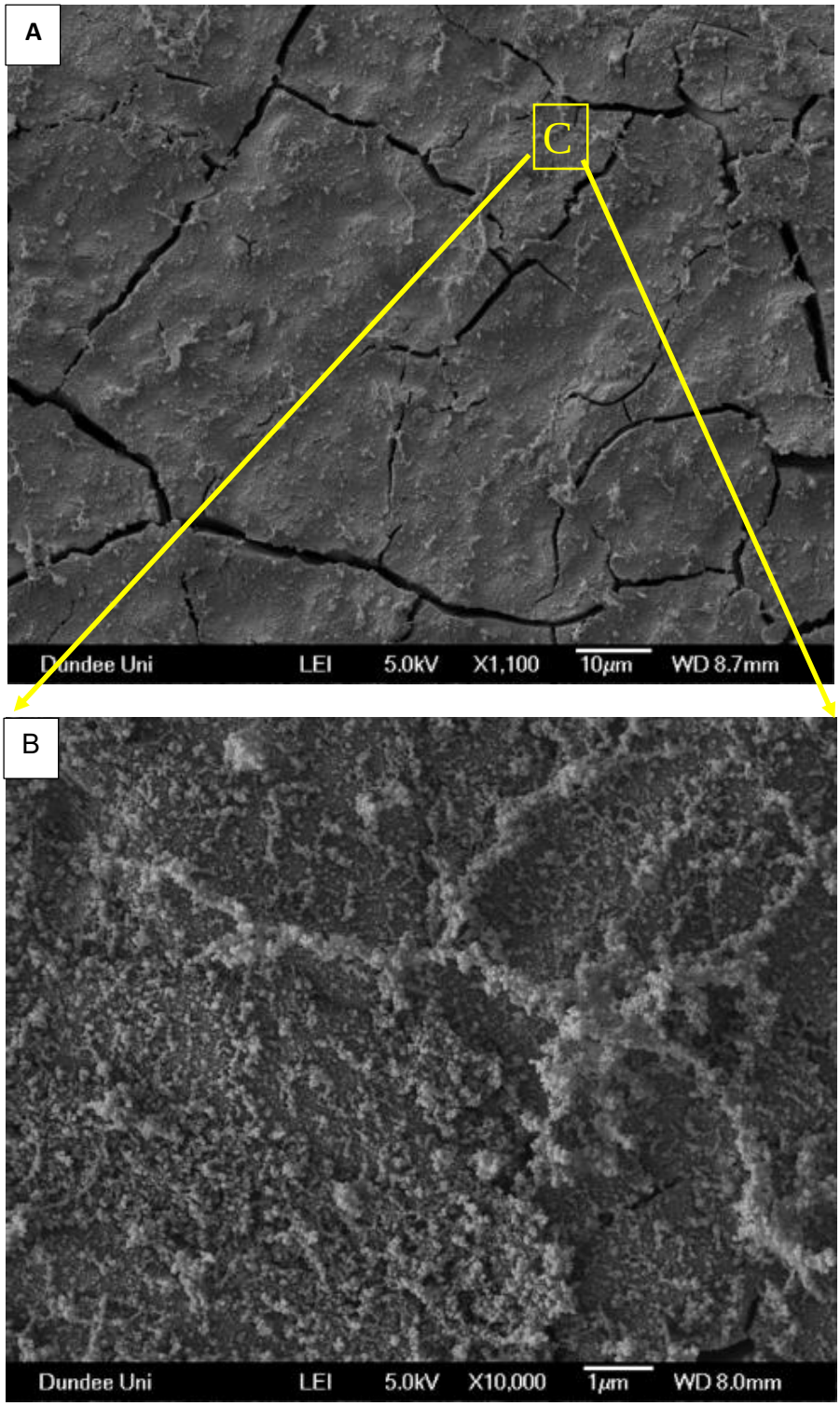


Figure 5.20 SEM images for unwashed 3D early coloniser biofilm on GIC disk, (A) is at 1100X magnification, while (B) is a higher magnification (10000X) of area C from image (A).

They show a dense bacterial biofilm covering GIC surface.

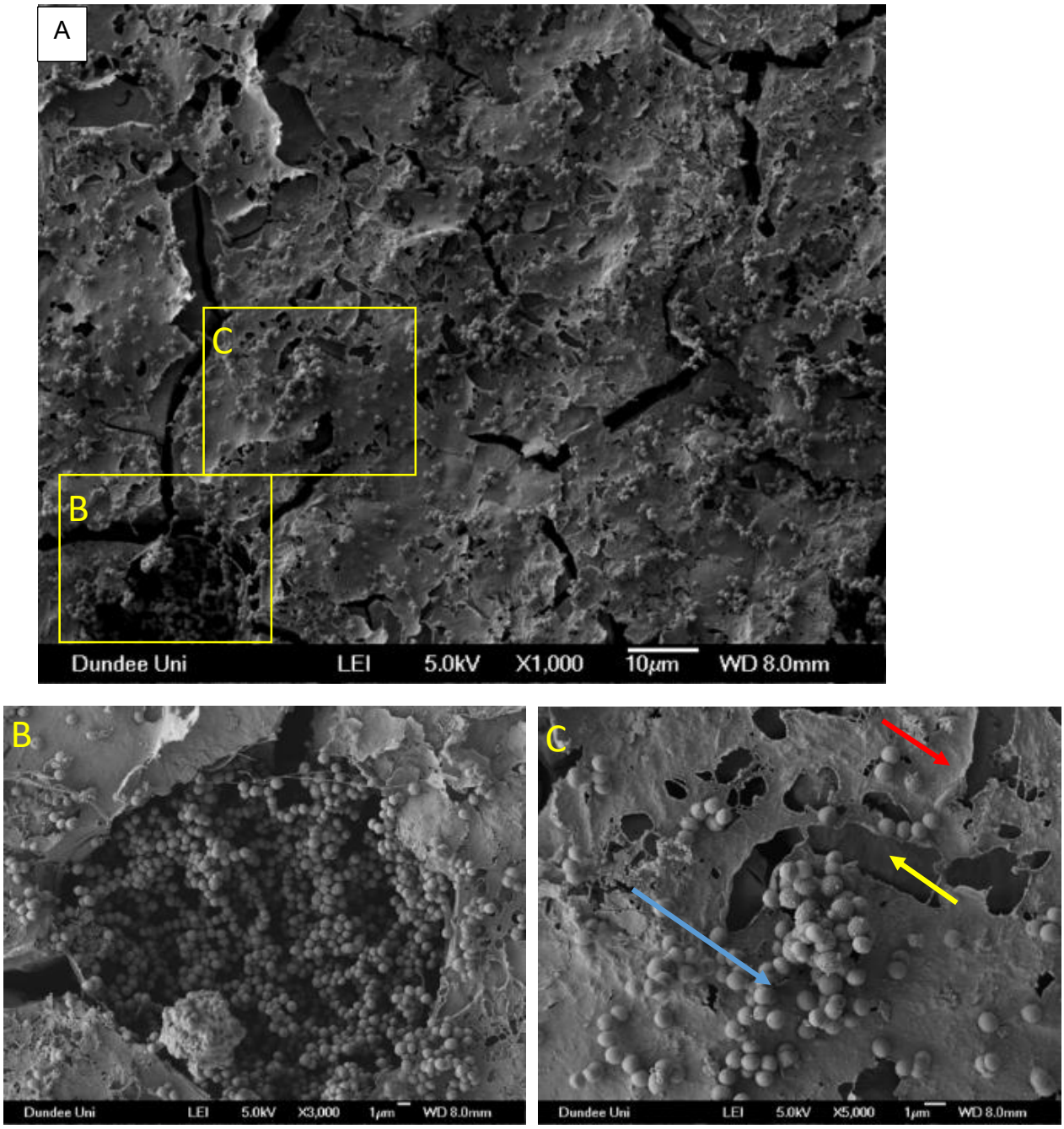


Figure 5.21 SEM images for 3D early coloniser biofilm on GIC disk after five times washing series, (A) at 1000X magnification while B and C are a higher magnification of areas B and C in from image A.

They show a detailed biofilm of oral colonisers intimately attached to GIC surface, from image C it is clear how the three species (blue arrow) colonies the surface and the EPS (red arrow) covering GIC (yellow arrow). Image B shows how the bacterial species colonise GIC surface cavities.

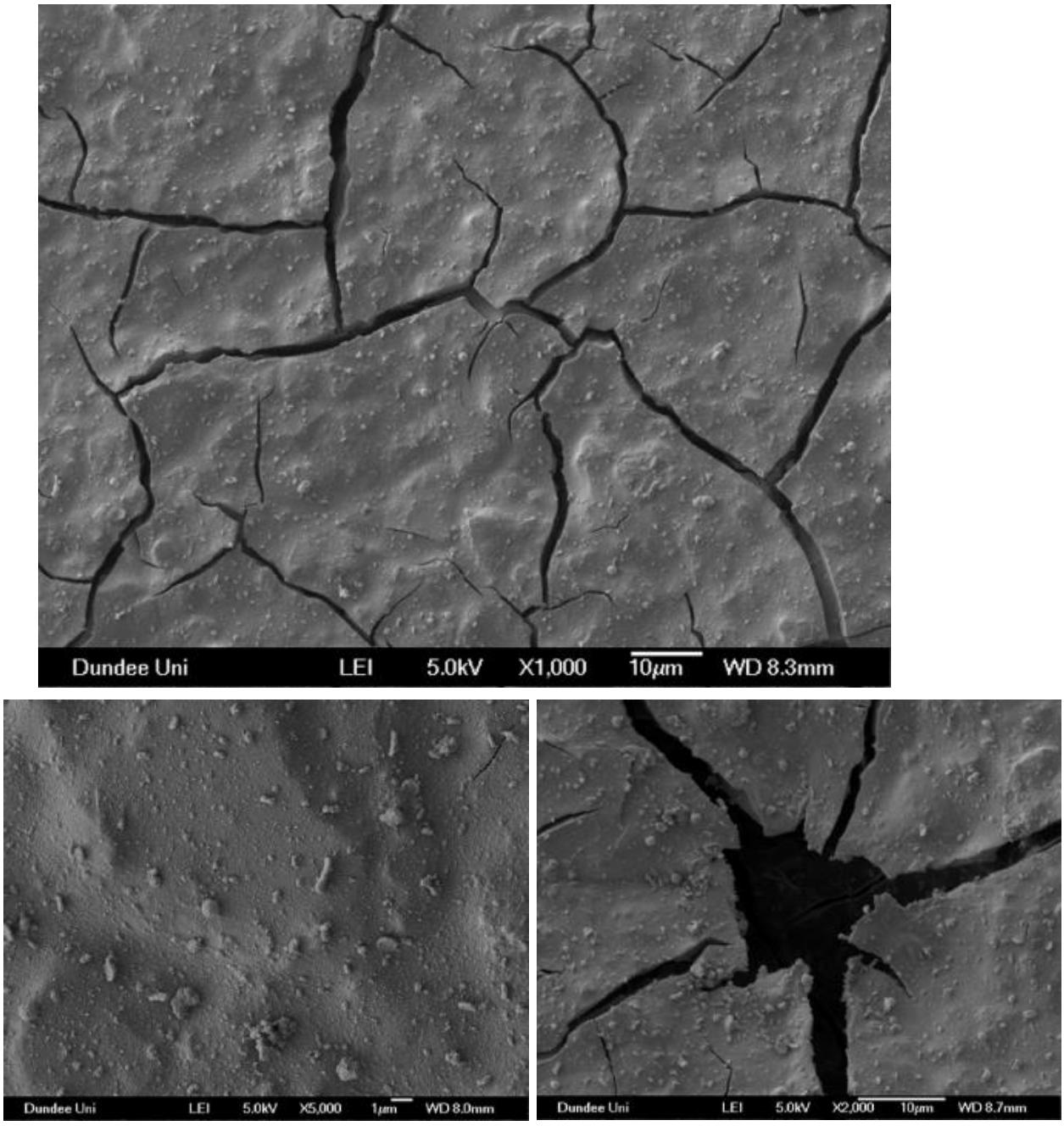


Figure 5.22 SEM images for GIC disk after sonication and vortexing at 1000X, 2000X and 5000X magnification, representing the efficiency of this method in detaching bacteria from GIC surface.

5.9.3 Discussion

From Figure 5.19, sonication and vortexing successfully managed to detach the three species from GIC. *S. oralis* was the predominant inhabitant with a mean total of 2.9×10^5 CFUs/ml. However, the total number of bacterial cells detached from GIC disks was on average 3.3×10^5 which is 100 times more than the fifth wash of five washing series (Figure 5.15).

From SEM images in (Figure 5.20), the unwashed 3D biofilm shows a heavy and dense colonisation with difficulty to define the features of the biofilm community. Compared to the washed 3D biofilm in (Figure 5.21) which shows more of the biofilm features and how the species colonise the surface. With higher magnification the fine feature of the three species could be examined and how they colonise the cavities of GIC surface (Figure 5.21A). EPS covering the GIC surfaces could be seen in Figure 5.21B. Because early coloniser model is concerned with the investigation of early colonisation, the washing step is important because it reduces the number of loosely attached bacteria in a controlled manner consequently the retained bacteria are assumed to have stronger adherence to GIC surface.

CFUs of the Intimately attached bacteria to GIC surfaces after washing represent a detailed and reliable count that could be compared through different conditions because washing step is a way of indirectly simulating the fluid flow of saliva, it is successfully reduced the overestimation effect of friable bacteria on results.

SEM images also showed that GIC surface almost free from bacterial cells, extracellular matrix and debris as shown in (Figure 5.22).

Within the parameters of this model, five washing series efficiently reduced the total number of loosely attached bacteria. In addition, sonication and vortexing method was an

efficient method for detaching *in vitro* biofilms from GIC surfaces after 24Hrs aerobic incubation.

5.10 Investigation of Molecular Techniques to Determine the Amount & Variety of Microbes Colonising Restorative Materials

The assessment of bacterial colonisation on glass ionomer disks over 48 hours in DMM artificial saliva had been developed as a first line in assessing the inhibitory effects of different additives. The use of selective media and determination of CFUs has some well characterised limitations, the most significant being the inability to distinguish between colonies formed by rafts of cells, one cell or chains of Streptococci whose cell walls have not completely separated (Figure 5.23).

Like all living things bacteria are characterised by having specific genetic material, consequently it was decided to investigate if the amount of bacteria associated with the examined restorative surface could be determined by assaying the concentration of DNA in samples, or even by counting the relative number of individual chromosomes using specific primers and quantitative PCR, a technique that would be valuable in determining the relative survival of species in more complex consortium models that involve more than three species i.e. the selective agar media technique would not be feasible with higher number of consortium species.

The relative size of the chromosomes is similar and an assumption that in slow growing biofilms the three species were mono-nucleate (contain a single chromosome) was confirmed by direct observation (data not shown).

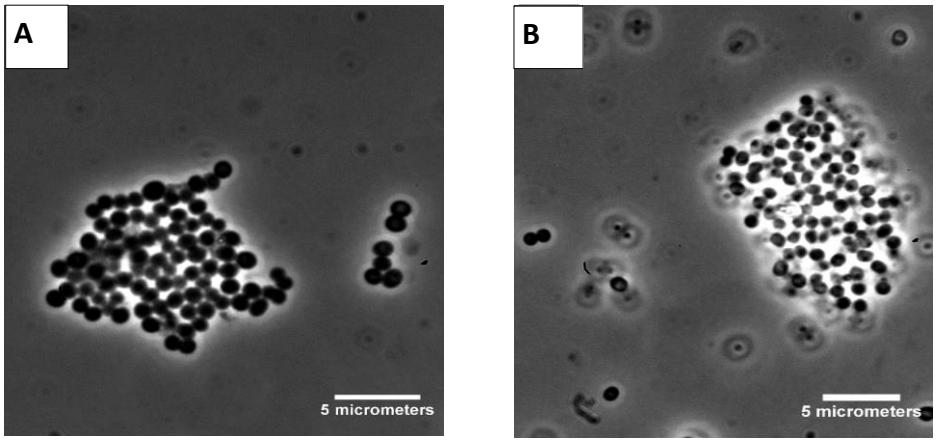


Figure 5.23 light microscopic images for *S. oralis* (A) and *S. mutans* (B) showing clumping of cells in broth cultures using DMM as growth medium.

5.10.1 Comparing the Efficiency of Two DNA Isolation Methods

Isolation of DNA from a range of samples is now commonplace and a variety of procedures have been adapted and mass produced in kit form. Initial work concentrated on quantitative isolation of DNA using one of these protocols, and a simplified physical method of extraction.

The DNeasy kit® is a commercially available DNA isolation kit which works through a membrane which combines the binding properties of a silica-based membrane with simple micro-spin technology. DNA adsorbs to the DNeasy membrane in the presence of high concentrations of chaotropic salt (can disrupt the hydrogen bonding network between water molecules) which remove water from hydrated molecules in solution. Buffer conditions in the procedure are designed to enable specific adsorption of DNA to

the silica membrane and optimal removal of contaminants and enzyme inhibitors. The harvesting of cells involves centrifugation followed by resuspension and lysis in high salt buffers supplemented with enzymes (Dilhari et al., 2017). The DNA is bound to the gel via a 'spin column' where the cell extract is loaded and then centrifuged using (Centrifuge 5415D, Eppendorf, Hamburg, Germany) at 14000 RPM. A series of washing steps and a final reversal of the pH to between 7-8.5 enables elution and concentration of DNA.

The advantage of this approach is its speed and the quality of DNA recovered which is suitable for reactions that include DNA sequencing. The second method is simply called Boiling technique, involved a simple harvesting step, and followed by resuspension in a buffer containing EDTA that can chelate cations which are involved in enzyme reactions that degrade DNA. The physical disruption of the cell was achieved by boiling for 10 minutes. Boiling is a common method for both inactivating and lysing bacteria (Dashti et al., 2009, Sahin et al., 2016).

5.10.1.1 Materials and Methods

Cultures in stationary phase (after 24Hrs incubation) for *S. mutans, S. oralis* and *N. subflava* in CB medium were prepared using the protocol described in section (5.1.1.2); their OD_{600} readings were 10.6, 16.5 and 5.7 respectively. Stationary cultures were chosen rather than mid-exponential because bacterial cells resulting after biofilm formation over restorative materials will be already in stationary phase after 24-48Hrs incubation.

Three different volumes from the same cultures were used to test if the varying amounts of cells and nucleic acid could be detected following the two extraction techniques. 1ml, 0.5ml and 0.25ml were collected from each culture into labelled Eppendorf tubes and then they were spin down at maximum speed to collect a pellet, theoretically pellets from 1ml samples should have double DNA amount of 0.5ml samples and 4 times more than 0.25ml samples. After that DNA was isolated using DNeasy Blood & Tissue Kits®

according to manufacturer's instructions which involve multiple steps of adding specific lysing enzymes, buffers and reagents, also involves spinning and centrifugation and finally DNA is captured by silica membrane and it is eluted by centrifugation.

Another set of cultures at same volumes and number were used to isolate DNA by Boiling method using the following protocol.

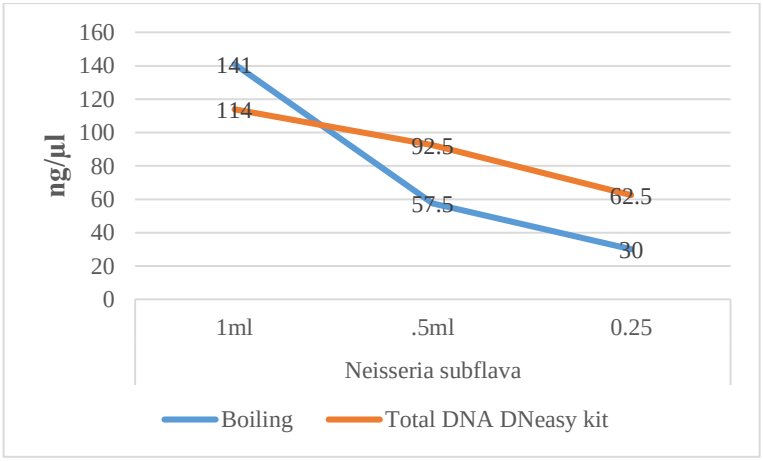
- Cultures were spin down for 3 minutes at 13000RPM speed, then the supernatant was discarded, after that pellets were re-suspended with 1.5ml of sterile water, then they were spin down as previous step. This step is for washing the pellet and it was repeated 3 times.
- The pellets were re-suspended finally with 1.5ml sterile water and the Eppendorf tubes were inserted in a shaking heat block at 99°C for 10 minutes. Before insertion the top of each tube was punctured with a tip of a needle to vent it and prevent pressure accumulation.
- After boiling, the tubes were left resting for 1 minute in ice then they were spin down for 3 minutes at 13000RPM speed, the supernatant was collected in labelled tubes which represent the total DNA; then the quantity and quality for DNA were measured using a Nano-drop spectrophotometer (Nanovue, General Electrics healthcare, Sweden).

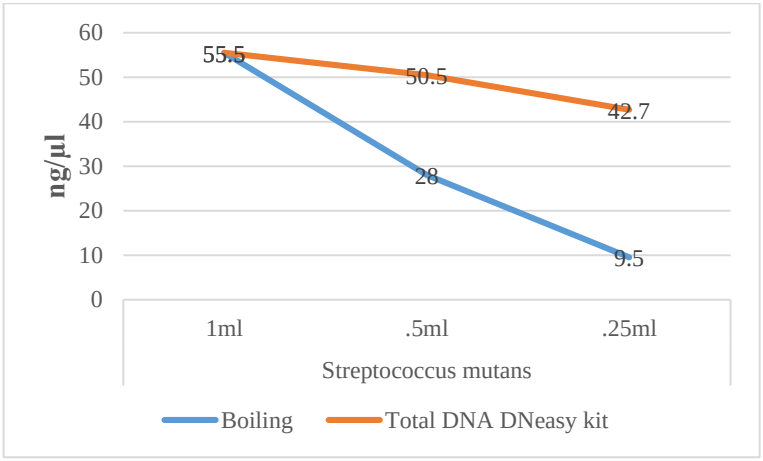
5.10.1.2 Results

In Table 5.6, DNA concentration and purity values resulted from each culture. Also graphs Figure 5.24 represent the relation between sample volume and quantity reduction using both methods, DNA quantities in graphs from DNeasy kit is the sum of the two elution.

Table 5.6 DNA quantity and purity yield from boiling and DNeasy® kit for three volumes of each species of the early coloniser model. First elute means the DNA eluted from first wash, and the second elute was extra optional method of the kit protocol.

		Boiling		**DNeasy first elute**		**DNeasy second elute**	
Species	**volume**	**Quantity ng/µl**	**Purity A260/280**	**Quantity ng/µl**	**Purity A260/280**	**Quantity ng/µl**	**Purity A260/280**
N. subflava OD_{600} = 5.7	1ml	141	2.2	80	2	34	1.9
	0.5ml	57.5	2.2	65	2.1	27.5	1.9
	0.25	30	2.1	47	2	15.5	1.8
S. mutans OD_{600} = 10.6	1ml	55.5	2.6	33.5	1.8	22	1.8
	0.5ml	28	2.3	21.5	1.7	29	1.8
	0.25ml	9.5	1.7	28.5	1.8	14.2	1.78
S. oralis OD_{600} = 16.5	1ml	121	2.6	216	1.5	489	1.43
	0.5ml	65	2.6	324	1.5	389.5	1.43
	0.25ml	37	2.3	244	1.46	300	1.42





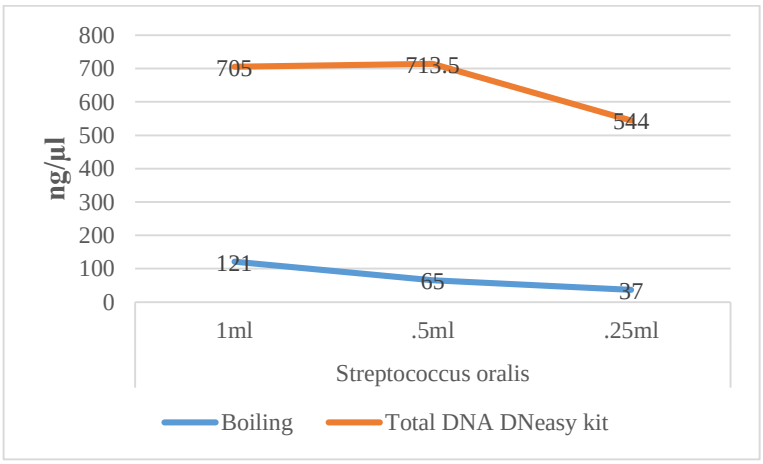


Figure 5.24 Graphs represent the relation between DNA quantity (ng/µl) isolated from each species of early coloniser model and the culture volume from which they were isolated using Boiling method versus DNeasy® kit, cultures were at stationary phase and OD_{600} is different between species. DNeasy Kit quantity in those graphs is the sum of both elutes.

5.10.1.3 Discussion

Theoretically, the bacterial DNA amount for a specific bacterial species derived from specific volume will be double the amount of the half volume at the same optical density. Assuming bacterial cells are well distributed in cultures and excluding the effect of clumping and chaining of bacteria on OD_{600} reading, cultures were thoroughly vortexed and checked microscopically for that reason.

However, DNA isolation using DNeasy Blood & Tissue Kits® is limited to the number of cells in the culture to be isolated which shouldn't be more than 2×10^9 CFUs/ml. for that reason DNeasy Blood & Tissue Kits® may not be suitable for isolation of early coloniser species model after incubation because they will be in stationary phase and number of bacterial cells either from planktonic growth or from wash assay may exceed that limit (Figure 5.11).

Comparing the isolation efficiency between Boiling and DNeasy Blood & Tissue Kits® of three cultures for each early coloniser species revealed the following:

Using the Boiling method, the relation between sample volume and the DNA quantity isolated, appeared to be related. The linearity of the graphs (Figure 5.24) indicates the expected reduction of nearly 50% in the amount of DNA when the volume halved. By contrast there appeared to be no specific relation between sample volume and DNA quantity isolated using DNeasy Kit. Indeed, sometimes the concentration increased for smaller volumes of harvested cells, for example DNA concentration of *S. oralis* culture from the first elute was 216µg/ml from 1 ml culture where it was 324µg/ml from 0.5ml cultures and 244µg/ml from 0.25ml culture which may be explained because total DNA elution was incomplete and DNA still binds to the membrane, that is clear from the amount of DNA from second elute (Table 5.6).

If the amount of second elute is added to the first elute result in Total DNA and then compared and related to volumes, the reduction still is not related to sample volume (Figure 5.24).

The most common purity calculation is the ratio of the absorbance at 260nm divided by the reading at 280nm. Good-quality DNA will have an A_{260}/A_{280} ratio of 1.7–2.0. A reading of 1.6 does not render the DNA unsuitable for any application, but lower ratios indicate more contaminants are present (Glasel, 1995).

The purity of DNA samples which is represented with A260/280 reading was different between the two methods. DNeasy Blood & Tissue Kits® used in isolation of *N. subflava* and *S. mutans* gave a good purity while in *S. oralis* the purity was less than 1.7 in the first and second elute. All A_{260}/A_{280} readings from Boiling methods was above 2 indicating the presence of contaminants RNA. In Boiling method RNA is not removed from specimens as it is in DNeasy kit, RNAse and proteinase are reagents that are used to digest and clean DNA in DNeasy kit. As a result, a second step of cleaning i.e. adding RNAse and proteinase may be needed for DNA produced from Boiling method which will render a purer sample.

DNA is not the only molecule that can absorb UV light at 260nm. Since RNA also has absorbance at 260nm, and the aromatic amino acids present in protein absorb at 280nm, both contaminants, if present in the DNA solution, will contribute to the total measurement at 260nm. This means that if the A_{260} number is used for calculation of yield, the DNA quantity may be overestimated (Manchester, 1995).

Boiling DNA isolation method is simpler and more effective than DNeasy Blood & Tissue Kits® in isolation of dense bacterial cultures but the resultant DNA required

further purification for high precision reactions such as sequencing and Quantitative PCR assay.

Within this project assay where the colonisation of the three species will be assayed over GIC and then its silver modifications, Boiling method with extra purification method would be more feasible, reliable and simpler than DNeasy Kit® in DNA quantification.

5.10.2 Identification of Reagents for the Counting of Chromosomes by qPCR

PCR amplifies specific regions of DNA that can be specific to individual species or across microbes by combined targets such as 16s RNA that offers a range of target gene, PCR can be used to analyse extremely small amounts of sample (Baker et al., 2003).

Quantitative PCR is an established tool for DNA quantification that measures the accumulation of DNA product after each round of PCR amplification. The method allows the estimation of the amount of a specific sequence present in a sample. A technique often applied to quantitatively determine levels of gene expression. By monitoring the amplification of a targeted DNA molecule at the end of each PCR amplification, i.e. in real-time, and not at its end, as in conventional PCR, more accurate quantitative information can be obtained (Alvarez et al., 2013).

There are two strategies for the Quantitative PCR, non-specific fluorescent DNA dyes and fluorescently labeled oligonucleotide probes. These two approaches were developed in parallel (Higuchi et al., 1992, Holland et al., 1991) and are used in pathogen detection; however, probe-based chemistry are dominating due to its higher specificity mediated by a probe which offers additional oligonucleotide, and the lower susceptibility to visualise non-specific PCR products, e.g. primer dimers (Bustin et al., 2009, Kubista et al., 2006). Quantitative PCR is carried out in a thermal cycler with the capacity to illuminate each

sample with a beam of light at least one specified wavelength and detect the fluorescence emitted by the excited fluorophore, section (4.6) for further details.

The specificity of PCR is supplied by the primers which target the polymerase and ensure the specific amplification of a product of known length and nucleic acid composition.

For determining the relevant amount of the three species used in the early coloniser model employed in this study it would be necessary to identify specific primer pairs that would selectively amplify the chromosome of in addition to targeting a gene sequence to be amplified that would be specific. It would also be important to establish that the primers did not cross react with other bacterial DNA sequences and generate non-specific products. To achieve this a set of preliminary PCR experiments that involved identifying the most promising target sequences (i.e. genes), designing appropriate primers for qPCR, & testing them on genomic DNA isolated by the DNeasy Kit®.

5.10.2.1 First Primers Set and PCR

The first step was to look for a specific gene for each species from the early coloniser model. Searching the literature for specific genes and primers, first set are listed in (Table 5.7). *S. mutans* is usually identified genetically by the presence of Glucosyltransferase enzyme genes (*gtfB*) which is responsible for the production of glucan and subsequently EPS (Busscher et al., 2010). *S. oralis* has a similar gene with a significantly different sequence called (*gtfR) (Hoshino et al., 2004). N. subflava* identified by 50S ribosomal protein L6 (*rplF*) gene which is specific to Neisseria spp. and used for their detection (Bennett et al., 2014).

Designing specific primers used to identify and quantify those species when they are in mixed community is important not only for this project but it may be used in another

occasions like for diagnosis and pathogenesis purposes or quantify them in saliva samples.

5.10.2.1.1 Methods and Materials

PCR reaction with primers listed in Table 5.7 was done using TopTaq DNA Polymerase® kit. First primers were ordered and then re-suspended using sterile distilled water following the manufacturer instructions. DNA samples from each species was quantified with spectrophotometer (Nanovue, General Electrics healthcare, Sweden) to determine their DNA concentrations; N. *subflava*: 43 ng/µl, *S. oralis*: 41 ng/µl and *S. mutans*: 19 ng/µl, all of them were isolated using DNeasy kit® from cultures at an $OD_{600}$of 0.1.

The reaction mixtures were prepared according to protocol recommended by the manufacturer, then they were inserted into thermal cycler (Figure 4.14) which was programed according to conditions in (Table 5.8) using protocol in section (4.6.3). After that a gel electrophoresis was conducted to PCR products according to methods in section (4.7) Figure 5.25 represent the reaction results.

Table 5.7 First list of primers names and the expected species which they are specific to, Primers forward (F) and reverse (R) sequences with their related annealing temperature at which the amplification starts. Also the expected product size in base pairs (bp).

Species	Target	Primer name	Sequence	Annealing temperature and Product size	source
S. mutans	16S-23S spacer	MUT-F MUT-R	CTCCTTTCTAAGGAAAAACGCA TGAACTCCAGACTGACTTATTAGAAAA	60 °C 388bp	Hoshino et. al. 2005
S. oralis* and *S. mitis	16S-23S spacer	O/M-F O/M-R	AGGATAAGGAACTGCACATTGGTC TGCATTACTTGGTGATCTCTCACC	68 °C 246bp	Hoshino et. al. 2005
N. subflava	rplF gene fragment	rplF-F rplF-R	CAGTGACTGTTCCCGCTGGTG AGGYTCAGGAGKWCGGAAHG	55 °C 413bp	Bennett et. al. 2014
S. mutans	gtfB gene	GTFB-F GTFB-R	ACTACACTTTCGGGTGGCTTGG CAGTATAAGCGCCAGTTTCATC	59 °C 517bp	Franco et. al. 2007

Table 5.8 First PCR conditions.

Steps	Time	Temperature
Initial denaturation	3 min	98 ºC
Denaturation	45 s	98 ºC
Annealing	30 s	60-52 ºC
Extension	50 s	72 ºC
Number of cycles	35	

5.10.2.1.2 Results

PCR products from gene amplification are presented as clear band and its size is related to the marker. Figure 5.25 represent gel electrophoresis for PCR products resulted from first set of primers, it can be noticed that;

rplF primer produced a product with the expected band size (413bp) from *N. subflava* DNA. However, there was unexpected product with *gtfB* primer and 16s-23s primer from *N. subflava* DNA.

Surprisingly *gtfB* primer amplified *S. oralis* DNA and gave a product with a right band size (517bp), that was opposite to what reported in the literature for that primer to be highly specific to *S. mutans* (Hoshino et al., 2004).

None of the other primers gave any product.

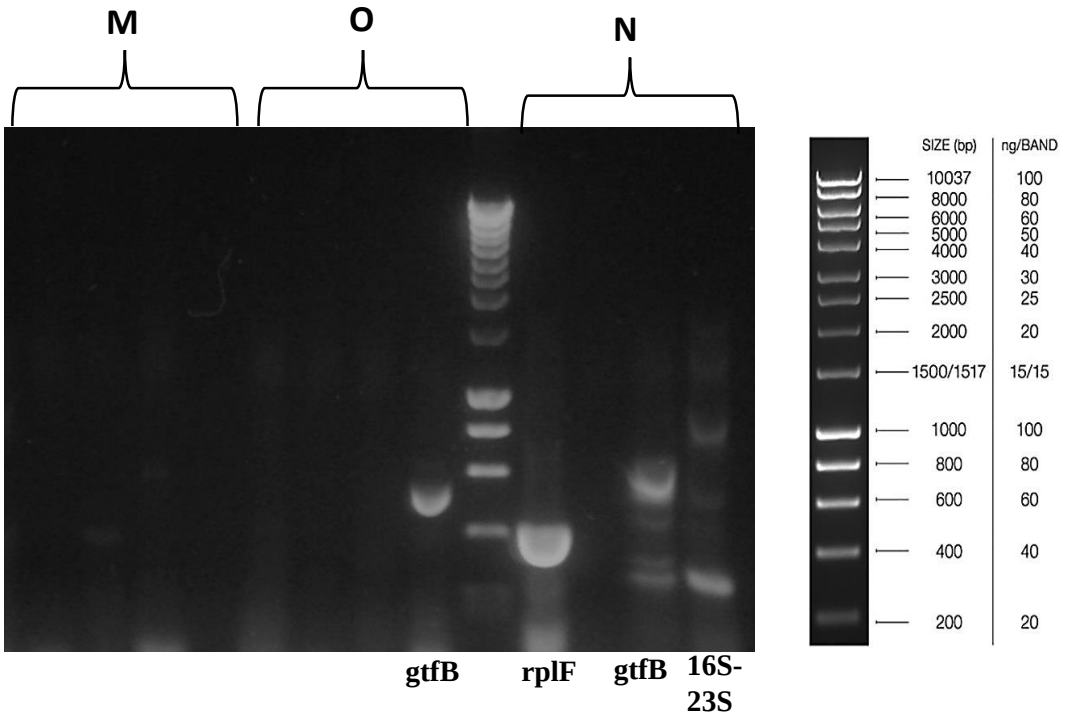


Figure 5.25 Gel electrophoresis for PCR products using first set of primers on *S. mutans* (M), *S. oralis* (O) and *N. subflava* (N) DNA samples.
a clear band related to *rplF* and two bands related to *gtfB* and 16S-23S resulted from *N. subflava* DNA. *gtfB* also amplified from *S. oralis* DNA. Size of bands identified by comparing the band produced with standard marker on the right.

5.10.2.1.3 Discussion

The expected size and product was obtained using *rplF* primers from *N. subflava* DNA, as a result this primer set showed specificity and was suitable for further development in qPCR assays. However the primers chosen for the *S. mutans gtf* gene that were reported to be specific, (Hoshino et al., 2004) cross reacted with the *N. subflava* DNA. Several products were observed with the *gtfB* primer set, including a strong band close to the expected size. This indicated that under the conditions used the specificity of these primers was compromised.

None of the primers listed successfully produced any product from *S. mutans* DNA despite another trials with fresh DNA samples or optimisations for the PCR conditions.

The universal primers pairs are meant to allow the identification of the total number of chromosomes as it should bind to conserved regions of the gene encoding the 16sRNA component of the Ribosome for all 3 species. In these initial experiments the 16s-23s primers, amplified only the *N. subflava* gene. This was completely unexpected, as they were chosen due to reported literature which claimed they would be highly specific to *S. oralis* and *S. mitis* (Hoshino et al., 2005). Analysis of deposited genomes suggested this would also be the case but it was noted that primers had not been used previously in mixed cultures.

For the ultimate aim of using qPCR reactions to count chromosomes, a robust set of specific PCR primers was required. The success of the *rplF* provided us with only one functioning set of reagents. Consequently, it was decided to identify alternative primers and try them alongside *rplF* and *gtfB* primers, and investigate minor modifications to PCR conditions that may increase specificity of primer binding to the level required. This involved using different annealing temperatures and implying a touch-down PCR to cycle across temperatures from highly specific to less specific (data not shown).

5.10.2.2 Second Primers Set and PCR

5.10.2.2.1 Martials and Methods

Primers listed in (Table 5.9), were tested. Same as previous experiment but conditions were slightly modified as seen in (Table 5.10). Specifically, the annealing temperature was lowered in the expectation that amplification from the *S. mutans* DNA would be obtained, and the extension time increased to ensure that the rate of polymerase processivity was in excess of that required, i.e. 90 seconds rather than the 30 seconds that would usually be used for a 500bp product.

The list included new universal primers (16s RNA universal) used to compare the amplification efficiency between DNA samples for the three species obtained from Boiling method and DNA samples obtained from DNeasy kit.

Table 5.9 Second list of primers names and the expected species which they are specific to, Primers forward (F) and reverse (R) sequences with their related annealing temperature at which the amplification starts. Also the expected product size in base pairs (bp).

Species	Target	Primer name	Sequence	Annealing temperature and Product size	Source
N. subflava	rplF gene fragment	rplF-F rplF-R	CAGTGACTGTTCCCGCTGGTG AGGYTCAGGAGKWCGGAAHG	55 ºC 413bp	Bennett et. al. 2014
S. mutans	GTF gene	GTFB-F GTFB-R	ACTACACTTTCGGGTGGCTTGG CAGTATAAGCGCCAGTTTCATC	59 ºC 517bp	Franco et. al. 2007
N. subflava ***S. mutans*** ***S. mutans***	16s RNA Universal	8f 1391R	AGAGTTTGATCCTGGCTCAG GACGGGCGGTGTGTRCA	52 ºC 797bp	Vickerman et al., 2007
S. mutans	kb -14 fragment	Sm479F Sm479R	TCGCGAAAAAGATAAACAAACA GCCCCTTCACAGTTGGTTAG	56 ºC 479bp	Chen et al 2007

Table 5.10 Second PCR conditions.

Steps	Time	Temperature
Initial denaturation	3 min	98 ºC
Denaturation	45 s	98 ºC
Annealing	30 s	60-50 ºC
Extension	90 s	72 ºC
Number of cycles	30	

5.10.2.2.2 Results and Discussion

Gel electrophoresis for the second set of primers PCR is presented in (Figure 5.26). It shows that, *rplF* again successfully amplifies the expected product with the expected band size (413bp). The modification to PCR conditions didn't affect the amplification of *gtfB* primer on *N. subflava* DNA nor *rlpF* primer on *S. mutans* DNA. However, it can be noticed from Figure 5.26 that increasing the extension time resulted in a 1.5 kb band, which suggests miss-priming was relatively non-specific as it resulted in multiple small bands (experiment 5.10.2.1) and an additional larger band (experiment 5.10.2.2). The possibilities for where the primers were binding on the *N. subflava* chromosomes was not easily detectable in silico (i.e. MacVector using *N. subflava* genome ATCC 49275). Consequently, the decision was to discount these primers rather than persist with further experiments.

Unexpectedly, *gtfB* primer failed to amplify *S. oralis* DNA which was opposite to previous experiment. Whether the lower annealing temperature, experimental error in loading reagents or the change in extension time had an unexpected impact remained

unclear. The key problem remained that a primer set described to be *S. mutans* specific was not performing in the expected manner for the three species we were examining.

Universal primers amplified a product from the three species DNA samples with the expected band size (797bp). Amplification from DNA prepared by boiling was less successful in terms of the amount of product that is reflected by bands density. In addition, amplification failed completely for *N. subflava*. All of that could be explained by the compromised purity of samples isolated by Boiling method compared to DNeasy Kit.

Boiling is crude quick method resulting in poor quality DNA samples which seemed to be not sufficient for predictable PCR results using standard PCR. Therefore, it was considered it would not be suitable for qPCR quantification, perhaps further purification could aid the evaluation of this material. However, it was not considered important while the process of identifying suitable qPCR primers was still on going. Amplification with universal primers seems to be unaffected with the quality of DNA resulted from Boiling method except in *N. subflava* DNA, which may be solved by cleaning up the samples with RNase and Protease. Another test is needed when the whole primers set are identified to validate using Boiling method in isolation of DNA in this project as if it is simpler than DNeasy Kit.

Despite the miss priming of *rplF* primer with *S. mutans* DNA, it can be assumed that it still specific to *N. subflava* depending on the intensity of the resulted band which is clearer than *S. mutans* (Figure 5.26). Overall it performed well with only a product appearing when the annealing temperature dropped below 52˚C, any final protocol would therefore employ a higher annealing temperature that ensured specificity to *N. subflava.*

gtfB however amplified *N. subflava* but not *S. mutans* which could be explained by technical or labelling error, the other assumption is *gtfB* is not highly specific to *S. mutans*

and to prove that further work was needed, but because these experiments were concerned in identifying specific primers for the three species, and their DNA samples were giving contradictory results to the published literature, it was considered important that the identity of the species was reconfirmed genetically. The products from universal primers amplification for the three species were purified using MinElute PCR Purification Kit® then the products were send for sequencing. To help confirm the identity of all the PCR products a decision was made to also sent the *rplF* from *N. subflava* and *gtfB* from *S. oralis* of the last experiment for sequencing.

The sequences obtained for the 16s RNA Universal primers products were analysed using several DNA genomic data base programs, with the SILVA high quality ribosomal RNA database proving the most effective at displaying genetic identity which resulted in 99.86 matching identity for all three species (Appendix 1 for figures and sequences).

Sequences from *rplF* and *gtfB* amplification were used to design qPCR primers, because as stated earlier qPCR conditions needs primers that give smaller product size than PCR primers, e.g. Roche® LightCycler primers machine needs primers with product less than 150bp.

Consequently, sequences from *rplF* and *gtfB* products were analysed using Roche® Universal Probe Library Assay Design Centre to check the availability of predesigned primers (https://lifescience.roche.com/en_gb/brands/universal-probe-library.html),

Successfully qPCR primer for *rplF* was found but not for *gtfB*.

In conclusion, the identity of the three micro-organisms was confirmed using universal 16s primers. And a qPCR primer for *N. subflava* were obtained.

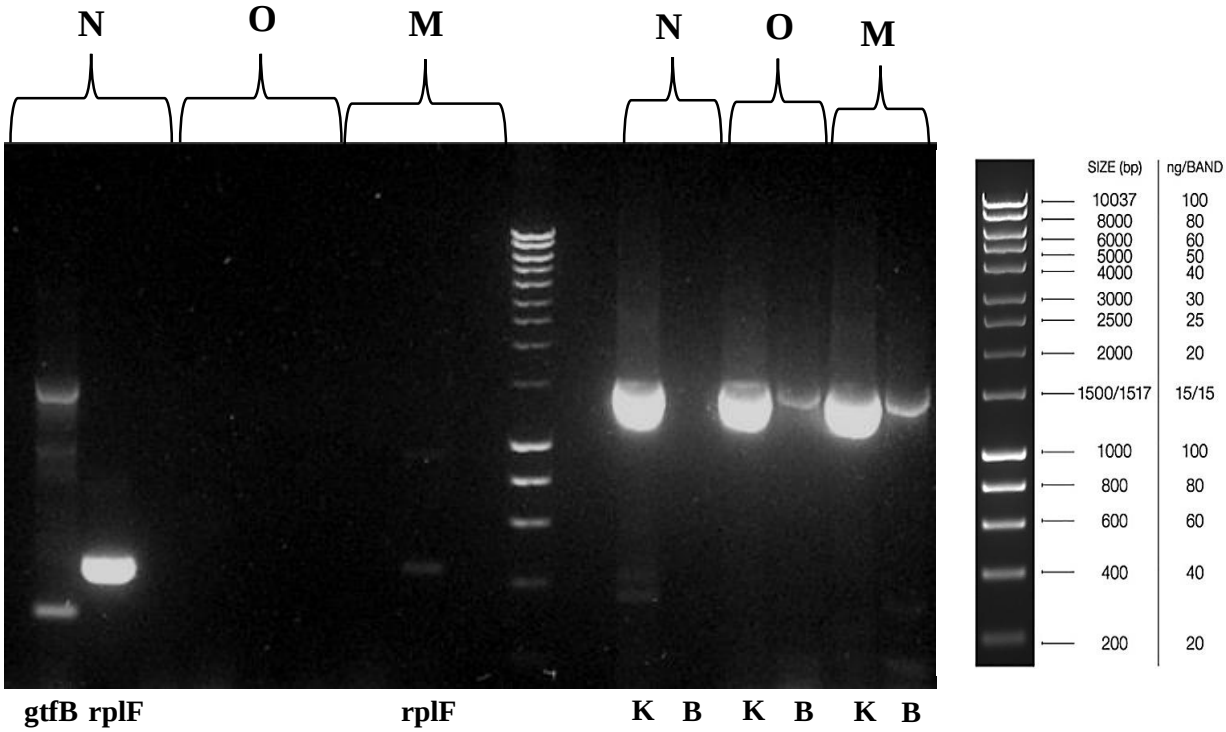


Figure 5.26 Gel electrophoresis for PCR products using Second set of primers on *S. mutans* (M), *S. oralis* (O) and *N. subflava* (N) DNA samples.

Bands to the right related to universal primer (16s universal) amplification of all DNA samples either from Boiling method (B) or DNeasy Kit (K) except *N. subflava* DNA from Boiling method. *rplF* amplified in *N. subflava* and *S. mutans* DNA. *gtfB* also amplified from *N. subflava* DNA. Size of bands identified by comparing the band produced with standard marker on the right.

5.10.2.3 Third Primers Set and PCR

5.10.2.3.1 Materials and Methods

Success with *rplF* primers in discriminating *N. subflava* & the ability to use universal 16s Primers to amplify sufficient sequence to confirm the identity of the three species was achieved in initial experiments. However, the ability of *gtfB* to discriminate *S. mutans* was not successful. In addition, multiple *N. subflava* products and the amplification of a product from *S. oralis* was inconclusive.

To confirm the amplification of *gtfB* gene on *S. oralis* DNA, fresh DNA samples for *S. mutans* and *S. oralis* were used after confirming their colony shape on MSA agar plates and their behaviour microscopically.

New primers with potential specificity were included after searching the literature alongside with *gtfB* primers from previous experiments (Table 5.11). *S. mutans* and *S. oralis* DNA samples were only used to run a PCR reaction flowing conditions in (Table 5.12).

Table 5.11 Third list of primers names and the expected species which they are specific to, Primers forward (F) and reverse (R) sequences with their related annealing temperature at which the amplification starts. Also the expected product size in base pairs (bp).

Species	Target	Primer name	Sequence	Annealing temperature and Product size	source
S. mutans	16S-23S spacer	MUT-F MUT-R	CTCCTTTCTAAGGAAAAACGCA TGAACTCCAGACTGACTTATTAGAAAA	60 °C 388bp	Hoshino et. al. 2005
S. oralis* and *S. mitis	16S-23S spacer	O/M-F O/M-R	AGGATAAGGAACTGCACATTGGTC TGCATTACTTGGTGATCTCTCACC	68 °C 246bp	Hoshino et. al. 2005
S. mutans	kb -14 fragment	Sm479F Sm479R	TCGCGAAAAAGATAAACAAACA GCCCCTTCACAGTTGGTTAG	56 ºC 479bp	Chen et al 2007
S. mutans	gtfB gene	GTFB-F GTFB-R	ACTACACTTTCGGGTGGCTTGG CAGTATAAGCGCCAGTTTCATC	59 °C 517bp	Franco et. al. 2007

Table 5.12 Third PCR conditions.

Steps	Time	Temperature
Initial denaturation	3 min	98 ºC
Denaturation	45 s	98 ºC
Annealing	30 s	60-52 ºC
Extension	30 s	72 ºC
Number of cycles	35	

5.10.2.3.2 Results and Discussion

PCR resulted gel electrophoresis is presented in Figure 5.27 which clearly shows that *gtfB* primer amplified a product from *S. oralis* DNA rather than *S. mutans* which was the same result from experiment 5.10.2.1. All the other primers used failed to produce a product from DNA samples tested. Having this product from *S. oralis* for the second time and not from *S. mutans* could be explained by the possibility of mutational changes in *S. mutans* genome and leads to a conclusion that *gtfB* primer is not highly specific to that strain from *S. mutans.* In addition, within the parameters of this experiment *gtfB* primers seemed to have specificity for *S. oralis* strain used in this project.

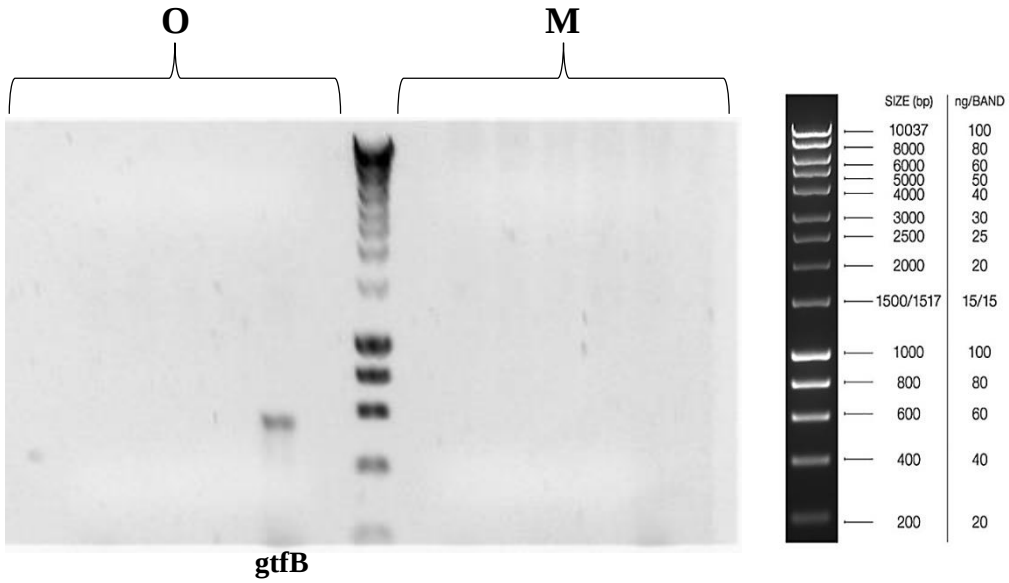


Figure 5.27 Gel electrophoresis for PCR products using Third set of primers on *S. mutans* (M), *S. oralis* (O) DNA samples only.

Only one product resulted which related to amplification of *gtfB* from *S. oralis* DNA. Size of bands identified by comparing the band produced with standard marker on the right.

5.10.2.4 Forth Primers Set and PCR

5.10.2.4.1 Materials and Methods

New primers with potential specificity to *S. oralis* and *S. mutans* were used (Table 5.13). Hashino et al (Hoshino et al., 2004) described the specificity of multiple *gtf* primers to multiple streptococci. As mentioned before most oral streptococci possess the glucosyltransferase (GTF) enzymes that use sucrose as a substrate to synthesise of EPS (Monchois et al., 1999), consequently that might help in finding a specific primer to *S. mutans* strain used in this project. To address this problem, the presence of *gtf* in *S. mutans* and *S. oralis* was investigated through PCR primers listed in (Table 5.13).

Table 5.13 Fourth list of primers names and the expected species which they are specific to, Primers forward (F) and reverse (R) sequences with their related annealing temperature at which the amplification starts. Also the expected product size in base pairs (bp).

Target	Name	Sequence	Annealing temperature	Product size
S.mutants *gtfD.*	MKD-F MKD-R	GGCACCACAACATTGGGAAGCTCAGTT GGAATGGCCGCTAAGTCAACAGGAT	70	433bp
S. oralis *gtfR*	MKR-F MKR-R	TCCCGGTCAGCAAACTCCAGCC GCAACCTTTGGATTTGCAAC	66	374bp

5.10.2.4.2 Results

Unfortunately, PCR reaction didn't reveal any products (Figure not shown).

5.10.2.4.3 Discussion

Control reactions using different primers confirmed that heat liable reagents including dNTPs were not compromised.

Species source from laboratory stocks has been explained in section 4.1.1.1. *S. mutans* and *S. oralis* were recovered from Dundee Dental School lab stocks, but the possibility of miss-assigned identity had been addressed by 16s RNA. This does no discount mutation, or strain variation however the failure of successive GTF primers was unexpected.

Despite the above speculations, the fact was the primers were not working. So the decision was to move on to another potential gene as it was unlikely that all the possibilities would fail.

5.10.2.5 Fifth Primers Set and PCR, qPCR

5.10.2.5.1 Materials and Methods

Rgg gene is part of *gtf* gene in *S. oralis* and it is proven to be highly specific in *S. oralis* identification (Park et al., 2010b). Consequently, *rgg* known gene sequence was aligned against the sequence resulting from *gtfB* amplification in the first experiment, primer pair were designed manually (Appendix 1 section 10.1.6). The resulted primers were supposed to be specific for *S. oralis*.

Regarding *S. mutans*, *gpsB* gene is guides peptidoglycan synthesis during the cell cycle and it is a cell division determinant in Gram-positive bacteria (Siegrist et al., 2015). *GpsB* known sequence for *S. mutans* was aligned against the know sequence of *S. oralis* then that alignment was used to design novel primers (Appendix 1 sections 10.1.7 and 10.1.8). It was decided to design new primers that fitted the more stringent requirements of qPCR. Briefly this involved loading the gene sequence from *S. mutans gpsB* into the Primer Designed portal hosted on the Roche® website (https://lifescience.roche.com/en_gb/brands/universal-probe-library.html).

This computer algorithm scans for primer pairs that fit criteria developed by the manufacturer & had previously proved to work well on other species of bacteria in the laboratory (data not shown).

All primers that were designed are listed in Table 5.14. In addition, qPCR primer for *rplF* gene was designed by Roche® data base and was included in the reaction.

PCR reaction was done for all the primers using conditions in (Table 5.15).

Table 5.14 Fifth list of primers names and the expected species which they are specific to, Primers forward (F) and reverse (R) sequences with their related annealing temperature at which the amplification starts. Also the expected product size in base pairs (bp)

Species	Primer name	sequence	Annealing temperature and Product size	Source
N. subflava	F-RlpNq R-RlpNq	GGGTTTCTCTCATCCAATCG GGCTAGGAGTTTGAACGGAAA	59˚C 62bp	Roche universal probe library
S. mutans	F-SMgpsB-q R-SMgpsB-q	AAGAGGCCGCTCAAACAAC GTCGCCACACGACTAGAACTT	60˚C 61bp	Designed manually
S. oralis	F-SOrgg-q R-SOrgg-q	TGAGGAGTGGGGACGTTATG AGCGTTTCCAAGGTCATCAA	60˚C 69bp	Designed manually

Table 5.15 Fifth PCR conditions.

Steps	Time	Temperature
Initial denaturation	3 min	98 ºC
Denaturation	45 s	98 ºC
Annealing	30 s	60-55 ºC
Extension	30 s	72 ºC
Number of cycles	30	

5.10.2.5.2 Results

Gel electrophoresis for PCR products is presented in (Figure 5.28).

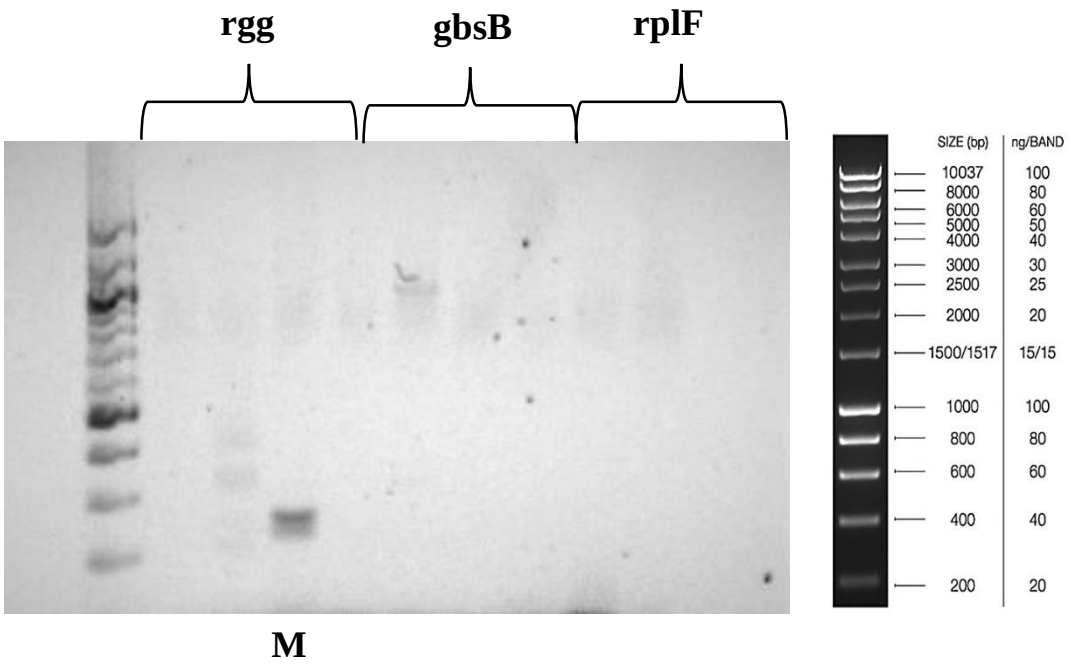


Figure 5.28 Gel electrophoresis for PCR products using Fifth set of primers on *S. mutans* (M), *S. oralis* and *N. subflava* DNA samples. Only one product resulted which related to amplification of *rgg* from *S. mutans* DNA. Size of bands identified by comparing the band produced with standard marker on the right.

5.10.2.5.3 Discussion

Surprisingly, *rgg* primer amplified *S. mutans* DNA rather than *S. oralis*. It may be because *rgg* is part of *gtf* gene which is a constituent of both species genome, miss priming and amplification might be expected. This finding was opposite to what has been proven that *rgg* primer is specific to *S. oralis* (Park et al., 2010b). Recombination & mutation of *gtf* gene might be beneficial for cariogenic organisms that need to have specific EPS in the

environment they can bind to & use for nutrition this hypothesis needs further investigation but that beyond the scope of this project.

Despite the challenging results obtained in standard PCR reactions, it was decided that screening the performance of the reagents using the specific qPCR mixture, and the micro titre plate format that is used by the Roches LightCycler® system we employed was worth investigating. qPCR assay on three species DNA samples using LightCycler® 480 control kit and FastStart® Essential DNA Green Master Kit according to the conditions in (Table 5.15) was conducted, *rplF* was the only primer amplified a product and it was only on *N. subflava* DNA samples (Appendix 1 section 10.1.9-10.1.11).

In conclusion, *rplF* primer seems to be highly specific to identify *N. subflava,* while further work is needed to identify the other primers. For reasons explained in section (6.5) this work was not going further and the biofilm was assayed with CFUs and MTT only.

5.11 Summary of Major findings

- DMM as a growth medium and artificial saliva, was capable of supporting the growth of the model species either aerobically or anaerobically up to 48 hours.
- Although the growth of early coloniser model species was slower in DMM than CB, DMM is still considered a rich saliva analogue especially in the presence of mucin and cystatins which favours biofilm formation in a more natural way. In addition, DMM contains a defined component superior to other artificial saliva recipes for example BMM (Wong and Sissons, 2001) allows changes to be made to its individual components, for example, to study factors regulating plaque growth and mineralisation.
- UV irradiation proved to be a suitable non-invasive method for sterilising Glass ionomer cements to be used in the *in vitro* model systems.
- A consistent and reproducible multispecies community of the early coloniser species in six well plate was established after 24Hrs using the three early colonisers growing in DMM. Furthermore, the early coloniser biofilm formed over a sterile GIC disks.
- An extra step of five times washing series allowed a more controlled comparison between tested samples, by eliminating the effect of friable bacteria on the end results. In addition, all samples were treated with the same conditions which reduce the effect of subjectivity and human errors.
- The growth and colonisation of the three species was assayed using CFUs for the growth around GIC disks (Floating), bacteria loosely attached to wells (Wash) and for the bacteria retained attached to disk surfaces after serial washing (Intimately attached).

Chapter 6 EXPERIMENTAL PART II (Silver Novel Additives to Glass-Ionomer Cement)

In this chapter the effect of silver additives to glass ionomer cement was investigated. The antibacterial activity was investigated first through comparing simple test methods to their antibacterial activity using the early coloniser model, second through comparing different incubation conditions and time frames of early coloniser model. However, silver solutions addition effect on the physical properties of glass ionomer cement was investigated via a set of standard physical tests that has a clinical relevance to the use of glass ionomer cement.

6.1 The Bacteriostatic and Bactericidal Activity of Silver Solutions against Early Coloniser Model Species in Solution (MIC/MBC)

The antibacterial activity of Silver solutions can be tested using simple methods, for example Agar diffusion test, MIC/MBC (Minimal Inhibitory Concentration/Minimal Bactericidal Concentration). Although these tests are widely used in the literature, when they are used to test the antibacterial activity of dental materials any results should be handled with care because they test the sensitivity of bacteria in broth culture or of a bacterial lawn in a Petri dish, consequently, results are not directive enough because of the difference between test conditions and oral environment.

This experiment was conducted to test the inhibition and killing of early coloniser model species in solution and then compare them with results from the final *in vitro* model.

6.1.1 Materials and Methods

6.1.1.1 Bacterial Species; *S. mutans* , *S. oralis* and *N. subflava*.

6.1.1.2 Growth Medium; CB.

6.1.1.3 Solutions; three silver solutions were chosen to be tested because of their stability compared to 22mg/ml silver solution which showed recrystallisation after 3 days of preparation, they were prepared following the protocols in section (4.8.3).

- Silver solutions; 5mg/ml, 13 mg/ml and 10mg/ml.
- PVA at 5mg/ml, 0.5M and 2.7M citric acid (citric acid concentration in 5mg/ml and 13mg/ml silver solution respectively) were used as a control because these materials were used to stabilise silver ions in their correspondence silver solutions consequently to test if they have additional antibacterial activity towards the early coloniser species.

6.1.1.4 Culturing Methods and Growth Conditions

A broth culture for each species was grown as described in section (5.2.1.3), but using CB as growth medium to obtain a 0.1 OD_{600} culture diluted from mid-exponential phase one. Then 200µl of each silver solution to be tested were added to 1.8ml of CB in 2ml size labelled Eppendorf tube (silver solution concentration in this labelled Eppendorf was 10 times less its normal concentration). Then a 1:10 dilution series in CB was made out of each labelled Eppendorf to dilute the concentration of each silver concentration 10 times at each step of the dilution series.

After that, 100µl of each species culture was added to each tube of dilution series, then they were incubated, one set aerobically and the other one anaerobically at 37°C while shaking at 350 rpm speed. After 24Hrs the tubes were visually inspected for turbidity (means bacterial growth), the last clear tube before the turbid one corresponds to the (MIC). From that clear tube and two consecutive clear tubes 100µl were plated out on CB plates and incubated aerobically for 24Hrs. Finally, the plates were inspected and checked for growth, the highest dilution that corresponded to plate with no bacterial growth represents the (MBC). This experiment was done three times each one is a month apart to test the longevity of bacterial activity over 3 months' period of time.

6.1.2 Results

Figures 6.1-6.4 represents graphs of the MIC/MBC on average (Mean±SEM) for each silver solution and 2.7M citric acid against the early coloniser species in solution.

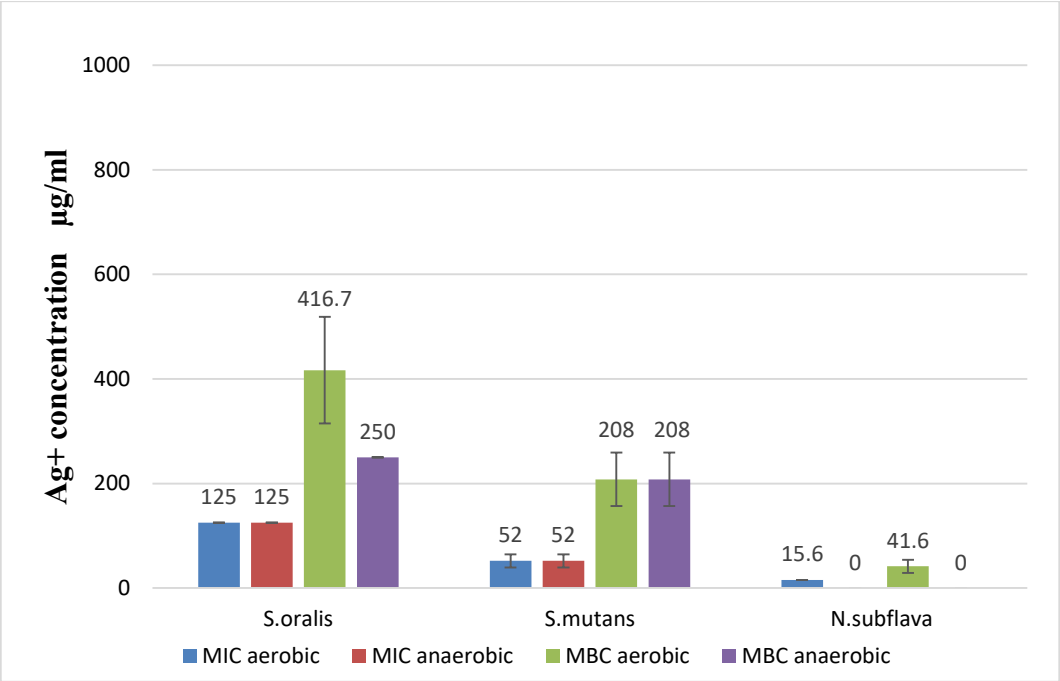


Figure 6.1 MIC/MBC average (Mean ± SEM *n* =3) in µg/ml for 5mg/ml Ag^{+} silver citrate/citric acid complex against *S. mutans, S. oralis* and *N. subflava* aerobically and anaerobically.

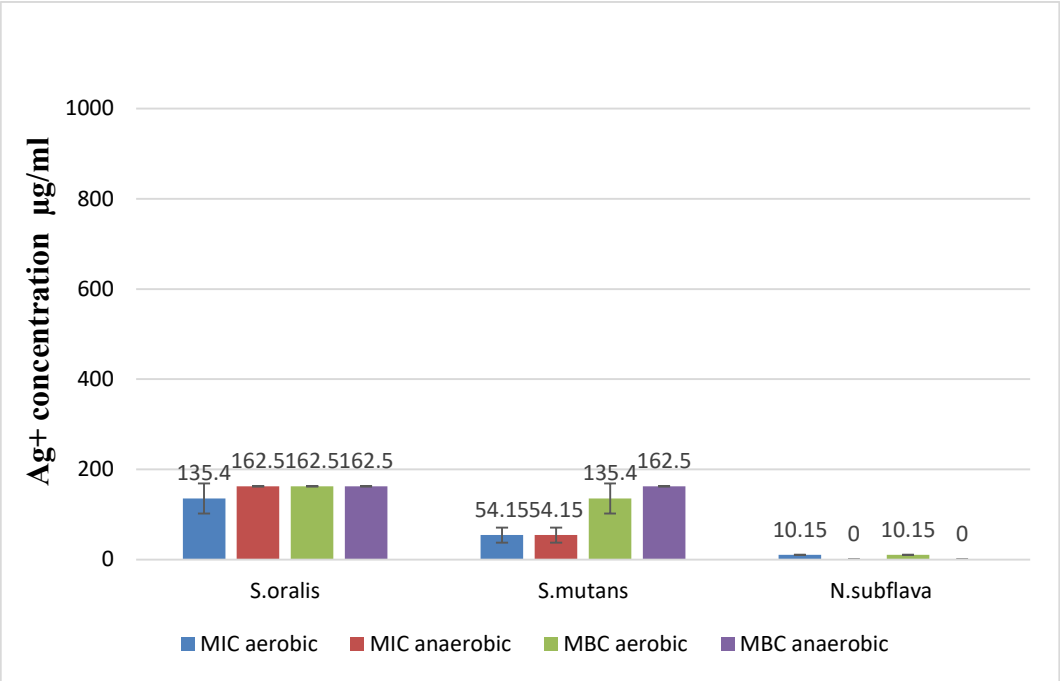


Figure 6.2 MIC/MBC average (Mean ± SEM *n* =3) in µg/ml for 13mg/ml Ag^{+} silver citrate/citric acid complex against *S. mutans, S. oralis* and *N. subflava* aerobically and anaerobically.

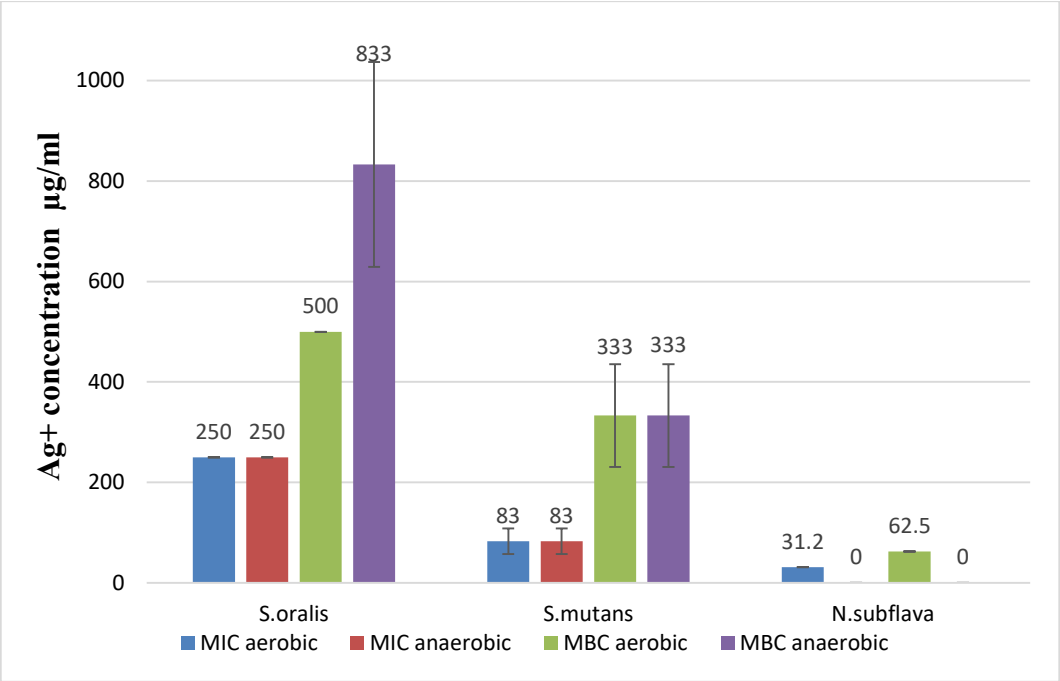


Figure 6.3 MIC/MBC average (Mean ± SEM *n* =3) in µg/ml for 10mg/ml Ag^{+} silver citrate/citric acid complex with PVA, against *S. mutans*, *S. oralis* and *N. subflava* aerobically and anaerobically.

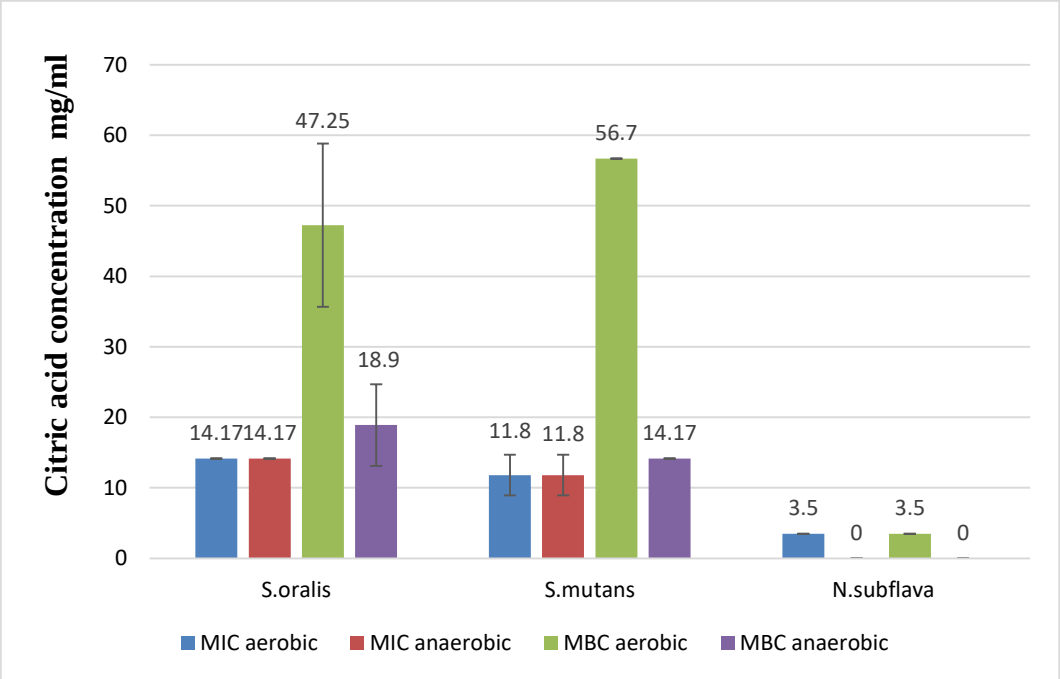


Figure 6.4 MIC/MBC average (Mean ± SEM *n* =3) in mg/ml for 2.7M citric acid against *S. mutans*, *S. oralis* and *N. subflava* aerobically and anaerobically.

6.1.3 Discussion

MIC is the lowest concentration of a chemical which prevents visible growth of a bacterium i.e. bacteriostatic activity. While MBC is the concentration resulting in microbial death i.e. the concentration at which it is bactericidal.

MIC/MBC are valuable tools to test the antibacterial activity in solution which is more important in cases of antibiotics in the medical field. But in cases of dental restorative materials, two aspects are important, first is the dynamics and complexity of the oral cavity which may adversely affect the lethal concentration of antimicrobials, for example saliva flow may wash out and dilute antimicrobials (Dawes et al., 2015). Second, the incorporation of antibacterial actives into restorative material may limit their dissolution in the oral cavity which result in different mechanism for that antimicrobial material to be effective.

In this experiment all silver solutions showed substantial antibacterial activity under both aerobic and anaerobic conditions, 13mg/ml silver solution showed the highest killing activity, aerobically 135.4μg/ml, 54.15μg/ml of it were needed on average to inhibit the growth of *S. oralis* and *S. mutans* , while *N. subflava* was more sensitive and needed 10.15 μg/ml to inhibit its growth (Figure 6.2). The MIC anaerobically were near aerobic reading 162.5 μg/ml and 54.15 μg/ml for *S. oralis* and *S. mutans* , but *N. subflava* because it is an obligate aerobic bacterium (Kaplan and Fine, 2002) didn't show any growth under anaerobic conditions (Figure 6.2). To confirm that single species cultures of *N. subflava* were done anaerobically without additives and showed negative growth.

MBC of 13mg/ml for *S. oralis* was similar under both conditions and was same as MIC anaerobically 162.5 μg/ml, also MIC was equal to MBC on average for *N. subflava* aerobically.

With 5mg/ml silver solution, MIC under both conditions was the same on average for both *S. mutans* and *S. oralis* . *N. subflava* was more sensitive than both streptococci. A higher concentration was needed to kill *S. oralis* aerobically than anaerobically, while the same concentration was needed to kill *S. mutans* in both conditions (Figure 6.1).

10mg/ml silver solution with PVA long chain linker, MIC under both conditions was the same on average for both *S. mutans* and *S. oralis* , also *N. subflava* was more sensitive than both streptococci. A higher concentration was needed to kill *S. oralis* anaerobically than aerobically, while the same concentration was needed to kill *S. mutans* in both conditions (Figure 6.1).

Comparing three concentrations, overall, *S. oralis* seems to be more resistant than *S. mutans* and *N. subflava* is the least. Aerobically 13 mg/ml silver solution appeared to have the highest killing activity on three species compared to 10mg/ml Ag^+ PVA and 5 mg/ml silver solution (Table 6.1).

13mg/ml silver solution contains 2.7M citric acid; this concentration shows antibacterial activity on the model species in solution (Figure 6.4), it kills *S. oralis* at 47.5mg/ml aerobically and 18.9mg/ml anaerobically. *S. mutans* was within the killing range of *S. oralis* , 56.7 mg/ml aerobically and 14.17mg/ml anaerobically. The same as other solution, *N. subflava* was more sensitive with MBC value of 3.5mg/ml which indicates that the acidity in 13mg/ml solution plays a role in the antibacterial activity of the silver solution.

0.5M citric acid presence in 5mg/ml silver solution seems to have negligible antibacterial effect as the case also of PVA which is present in 10mg/ml silver solution, because there was no inhibition in growth in all dilution series tubes.

Compared with other studies, silver nanoparticles (AgNPs) were mainly studied rather than silver solutions and complexes (Correa et al., 2015), their antibacterial activity is discussed in detail in literature review section (2.5.2). Only one study compared the antibacterial activity of sliver nitrate nanoparticles and silver citrate complexes in solution against *Pseudomonas aeruginosa* cultures (Djokic, 2008) They found that silver citrate/citric acid solution exhibited superior bactericidal activity over silver nitrate solution, a log reduction of 7.39 was achieved with silver citrate/citric acid solution which in that study suggesting a total kill of the tested organism compared to a log reduction of 0.16 after 30 minutes exposure to AgNPs.

MIC/MBC of AgNPs in previous studies against *S. mutans* and *S. oralis* is summarised in Table 6.2. Although AgNPs MIC and MBC are lower than results of this study which is related to variation in test methods, the stability and sustainability of AgNPs is really an issue. Their formation is quiet technique sensitive and they are susceptible to co-aggregation with time, despite the trials to stabilise them with organic polymers like PVA (Abou El-Nour et al., 2010, Pal et al., 1997).

The stability of silver solutions and sustainability of their antibacterial activity were tested by comparing MIC/MBC each month the experiment was conducted, 13mg/ml silver solution showed some recrystallisation and precipitation after 3months but retained its potent antimicrobial activity, 5gm/ml silver solution was also stable for 6 months, while 10mg/ml Ag^{+} PVA showed the best stability which lasted over 6 months.

In conclusion, three silver solutions showed potent antibacterial activity against early coloniser model species in solution, 13mg/ml silver solution had the highest antibacterial activity followed by 5mg/ml silver solution and lastly 10mg/ml silver solution. Part of the

killing activity of 13mg/ml was related to its high acidity because it contained 2.7M citric acid which was needed to solubilise silver citrate during its formation method.

Table 6.1 Mean MBC in µg/ml of three silver solutions on the early coloniser species in solution aerobically and anaerobically after 24Hrs.

Aerobically			
Species	**13mg/ml Ag^+**	**5mg/ml Ag^+**	**10mg/ml Ag^+ PVA**
S. oralis	162.5	416.7	500
S. mutans	135.4	208	333
N. subflava	10.15	41.6	62.5
Anaerobically			
S. oralis	162.5	250	833
S. mutans	162.5	208	333

Table 6.2 MIC/MBC for AgNPs dispersal solutions against *S. mutans* and *S. oralis* in previous studies.

Species	**AgNPs size nm**	**MIC µg/ml**	**MBC µg/ml**	**Source**
S. mutans	0.0976-100	4.86 ± 2.71	6.25	(Hernandez-Sierra, 2008)
	50-60	25	25	(Perez-Diaz, 2015)
	5	50	-----	(Lu et al., 2013)
	<100	50	-----	(Besinis et al., 2014)
S. oralis	5	50	-----	(Lu et al., 2013)

6.2 The Effect of Silver Additives on the Attachment and Survival of Early Coloniser Model under Aerobic Conditions after 24 Hours

After the antibacterial activity of silver solutions was tested simply by MIC/MBC assay, their effect on the colonisation of the early coloniser model after 24 Hrs incubation was tested after they were added to GIC. First experiment conducted under aerobic conditions.

6.2.1 Materials and Methods

6.2.1.1 Bacterial Species; *S. mutans* , *S. oralis* and *N. subflava*.

6.2.1.2 Growth Medium; CB and DMM.

6.2.1.3 Solutions; Silver solutions; 5mg/ml, 13 mg/ml and 10mg/ml.

6.2.1.4 Specimens Preparation

Silver modified GIC;

GIC powder (ChemFil Superior®) was mixed with each of the silver solutions, the volume needed from 5mg/ml and 13mg/ml silver solutions was the same volume of the water recommended by the manufacturer to prepare non-modified GIC. But with 10mg/ml silver solution which contains PVA, to reach the same non-modified GIC mixture consistency the volume increased by 50%. Silver modified GIC disks were formed using PTFE molds as in section (4.1.3.1). And the result shown in (Figure 6.5).

6.2.1.5 Culturing Methods and Growth Conditions

It was the same as in section (5.9.1.3), except in the arrangement of modified and non-modified disks through the wells; for each experiment 12 disks were tested, 3 for each silver solution and 3 non-modified GIC. 3 wells left as control contained the early coloniser species without any disk in. 6 well plates were incubated aerobically at 37ºC for 24Hrs.

The planktonic growth around disks (Floating), what was loosely attached to wells surfaces (Wash) and what remained attached to disk surfaces after 5 times serial washes (Intimately attached) was assayed using CFUs. The experiment was repeated 3 times.

Disks after washing and disks after sonication and vortexing were fixed and prepared for SEM using the protocol described in section (4.5.2) then were checked with SEM. For all SEM samples, the middle of each disk was used to have images because it's supposed to be least effective with handling and processing errors, 3 fixed areas were selected from each disk and through the other samples.

6.2.1.6 Statistical Analysis

For statistical analysis Friedman's two-way ANOVA by rank test was conducted to evaluate differences between the groups, a P value of < 0.05 was considered significant.

6.2.2 Results

The resultant GIC colour after silver solution addition is demonstrated in Figure 6.5, also shown is how they are positioned in the wells to form the *in vitro* biofilm in (Figure 6.6) in addition 13mg/ml silver addition to GIC failed to form stable formulation as shown in the same figure.

Figures 6.7-6.9 demonstrate the three outputs from wells using CFUs assay. The planktonic growth around disks (Floating), what was loosely attached to the well surfaces (Wash) and what remained attached to disk surfaces after 5 times serial washes (Intimately attached) for silver modified and non-modified GIC. 13mg/ml silver modified disks failed to give results because of their unstable formulation after incubation using the earl coloniser model.

Figures 6.10 and 6.11 are SEM images for the Intimately attached biofilm and validation of sonication and vortexing.

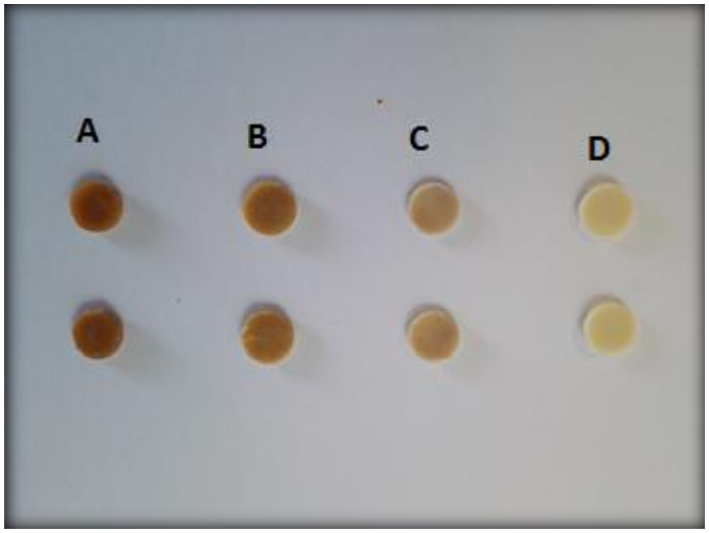


Figure 6.5 GIC disks modified with silver solutions, A: 13mg/ml Ag^{+}, B: 10mg/ml Ag^{+} PVA, C: 5mg/ml Ag^{+}, D: non-modified GIC**.**

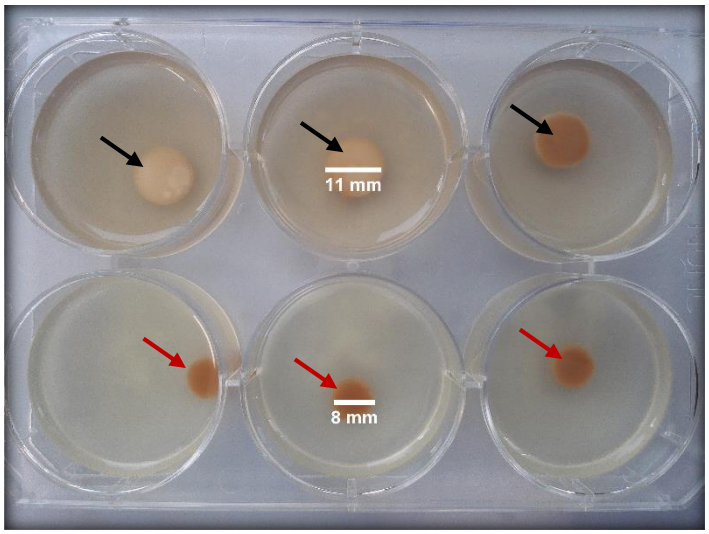
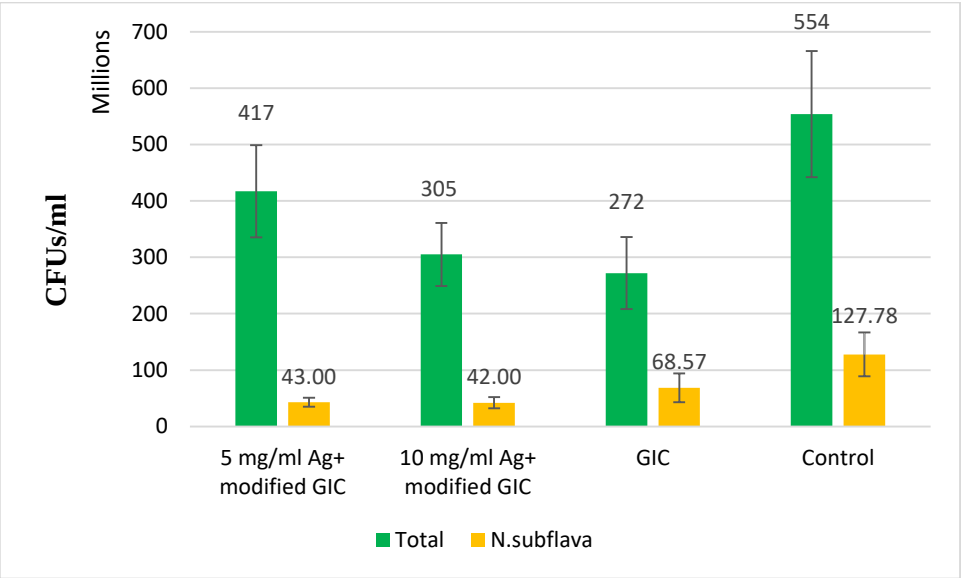


Figure 6.6 Six well plate with silver modified GIC disks after 24 Hrs incubation with early coloniser model species. The expansion of disks modified with 13mg/ml silver solution (black arrows) compared to 10mg/ml silver modified disks (red arrows).



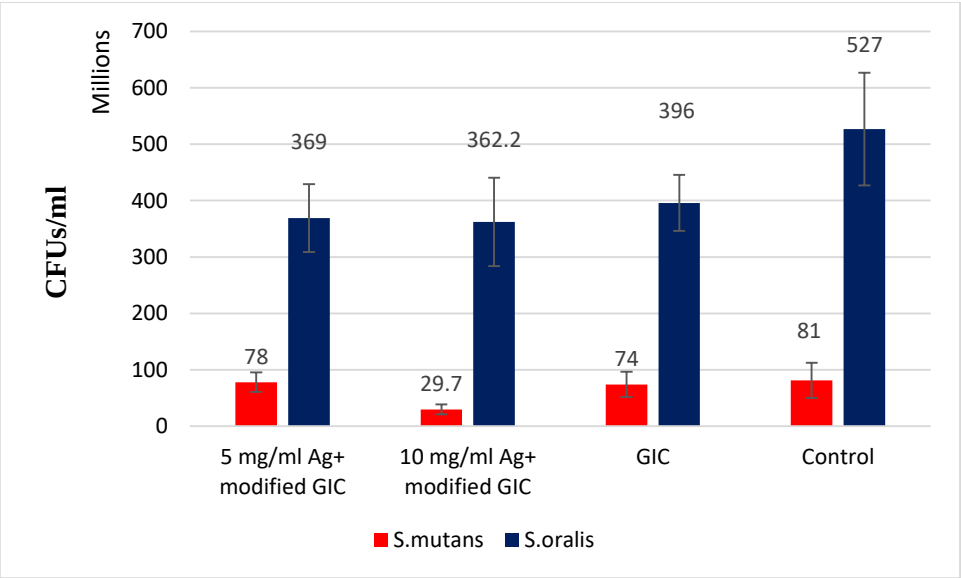
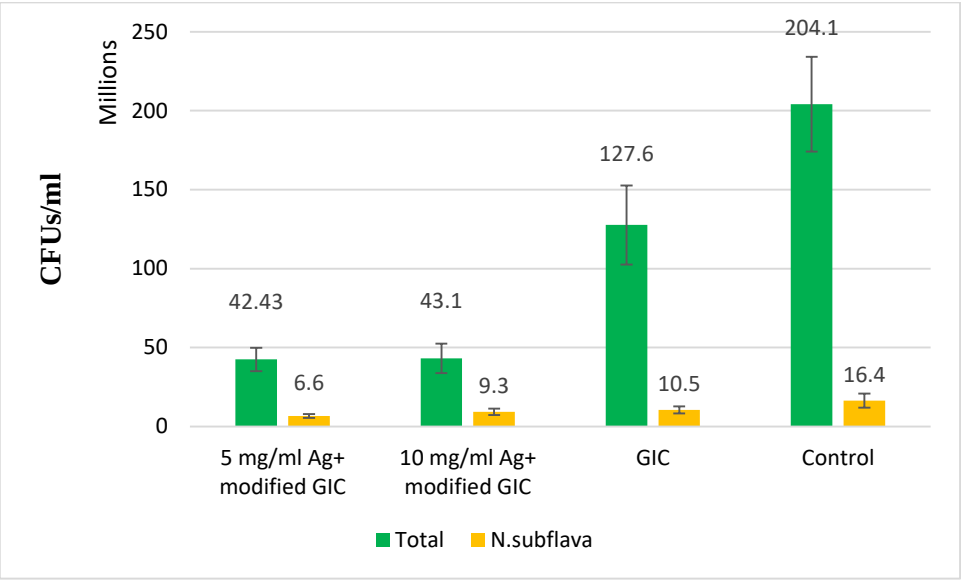


Figure 6.7 Graphs represent viable bacterial count (CFUs) (Mean ± SEM *n* =9) in millions for the planktonic bacterial growth around disks either silver modified or non-modified (Floating) in Total and for each of the three species after aerobic incubation for 24 Hrs. *S. mutans* means was significantly different between groups (Friedman's two-way ANOVA by rank test) ($p < 0.05$).

Table 6.3 Pairwise comparison of the *S. mutans* Floating CFUs showing a significant difference between wells containing 10mg/ml silver modified GIC in comparison to 5mg/ml silver modified GIC (* *p <0.05)*.

Sample1-Sample2	Significance
10mg/ml silver modified GIC – Control	0.414
10mg/ml silver modified GIC – ChemFil Superior GIC	0.226
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	***0.044****
Control – ChemFil Superior GIC	1.000
Control – 5mg/ml silver modified GIC	1.000
ChemFil Superior GIC - 5mg/ml silver modified GIC	1.000



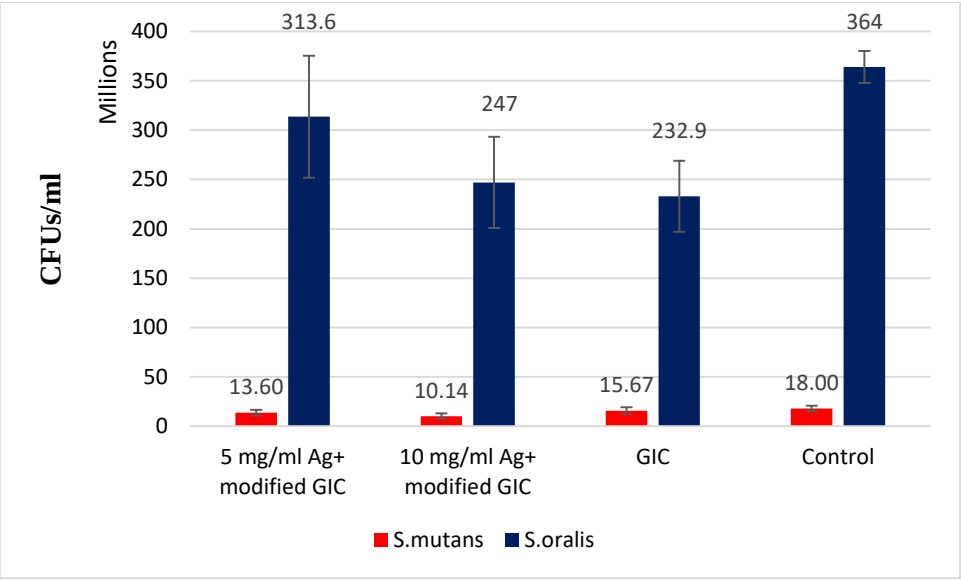


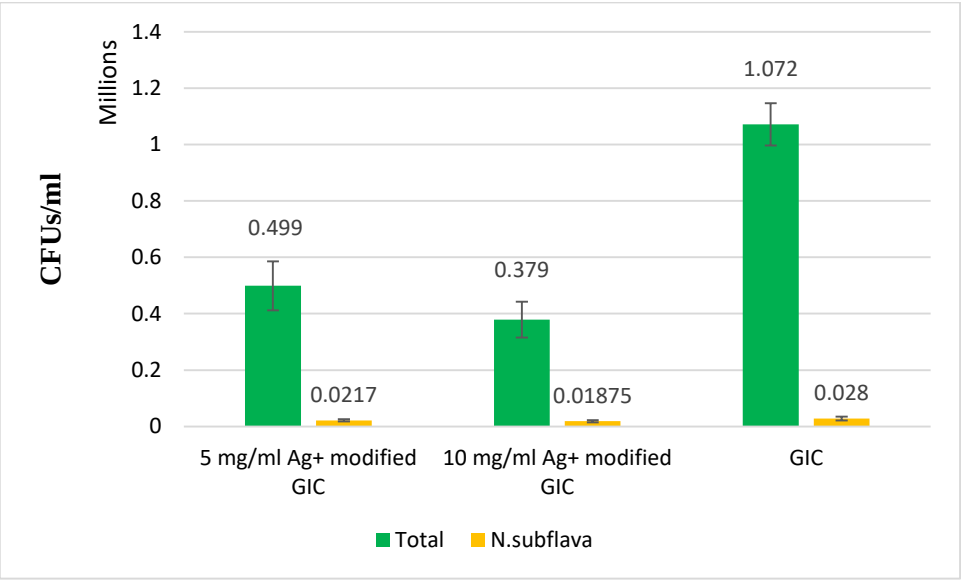
Figure 6.8 Graphs represent viable bacterial count (CFUs) (Mean ± SEM *n* =9) in millions in Total and for the three species assayed from wells surfaces washing (Wash) after aerobic incubation for 24 Hrs. Wash Total CFUs from both silver modified GIC disks containing wells was significantly lower than control, none of the rest were significantly different (Friedman's two-way ANOVA by rank test) ($p < 0.05$).

Table 6.4 Pairwise comparison of the Total Wash CFUs showing a highly significant difference between wells containing 10mg/ml silver modified GIC in comparison to 5mg/ml silver modified GIC, and in wells containing 5mg/ml silver modified GIC in comparison to control wells (* *p <0.05).*

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	1.000
10mg/ml silver modified GIC – ChemFil Superior GIC	1.000
10mg/ml silver modified GIC – Control	***0.001****
5mg/ml silver modified GIC – ChemFil Superior GIC	1.000
5mg/ml silver modified GIC – Control	***0.006****
ChemFil Superior GIC - Control	0.056

Table 6.5 Pairwise comparison of the *S. oralis* Wash CFUs showing no significant difference between wells containing silver modified and non-modified GIC (*p <0.05*).

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	0.995
10mg/ml silver modified GIC – ChemFil Superior GIC	1.000
10mg/ml silver modified GIC – Control	0.072
5mg/ml silver modified GIC – ChemFil Superior GIC	0.846
5mg/ml silver modified GIC – Control	1.000
ChemFil Superior GIC - Control	0.056



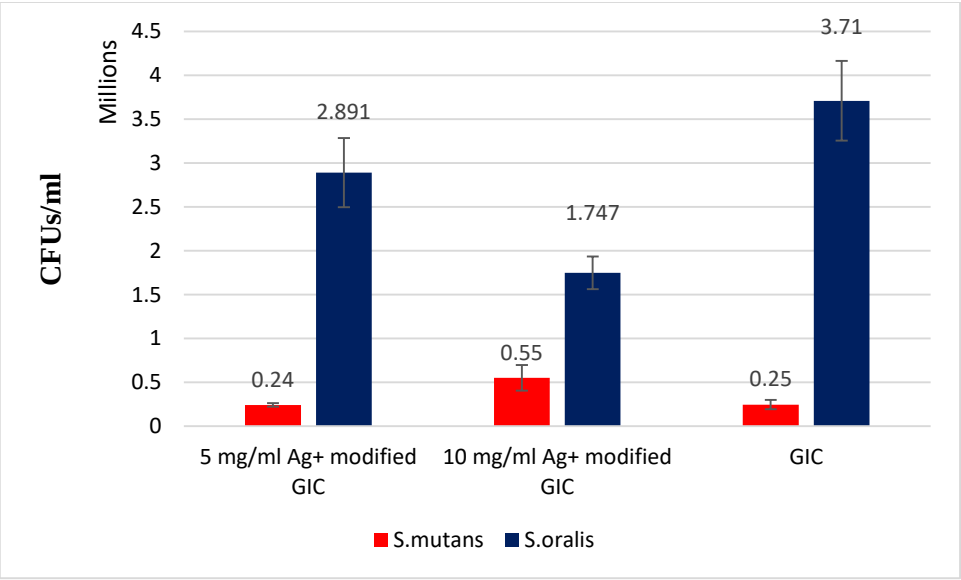


Figure 6.9 Graphs represent viable bacterial count (CFUs) (Mean ± SEM *n* =9) in millions in Total and for the three species (Mean ± SEM) assayed from disk surfaces (Intimately Attached) either silver modified or non-modified after aerobic incubation for 24 Hrs. Total CFUs from silver modified GIC disks were significantly lower than non-modified GIC, and *S. oralis* CFUs from 10mg/ml silver modified GIC disks was significantly lower than non-modified GIC disks (Friedman's two-way ANOVA by rank test) ($p < 0.05$).

Table 6.6 Pairwise comparison of the Total Intimately Attached CFUs showing a highly significant difference between wells containing 10mg/ml silver modified GIC in comparison to non-modified GIC, and in wells containing 5mg/ml silver modified GIC in comparison to non-modified wells (*$p < 0.05$).

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	0.791
10mg/ml silver modified GIC – ChemFil Superior GIC	***0.001****
5mg/ml silver modified GIC – ChemFil Superior GIC	***0.042****

Table 6.7 Pairwise comparison of the *S. oralis* Intimately Attached CFUs showing a highly significant difference between wells containing 10mg/ml silver modified GIC in comparison to non-modified GIC (* $p < 0.05$).

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	0.133
10mg/ml silver modified GIC – ChemFil Superior GIC	***0.002****
5mg/ml silver modified GIC – ChemFil Superior GIC	0.539

Non-modified GIC	5mg/ml Silver modified GIC	10mg/ml Silver modified GIC

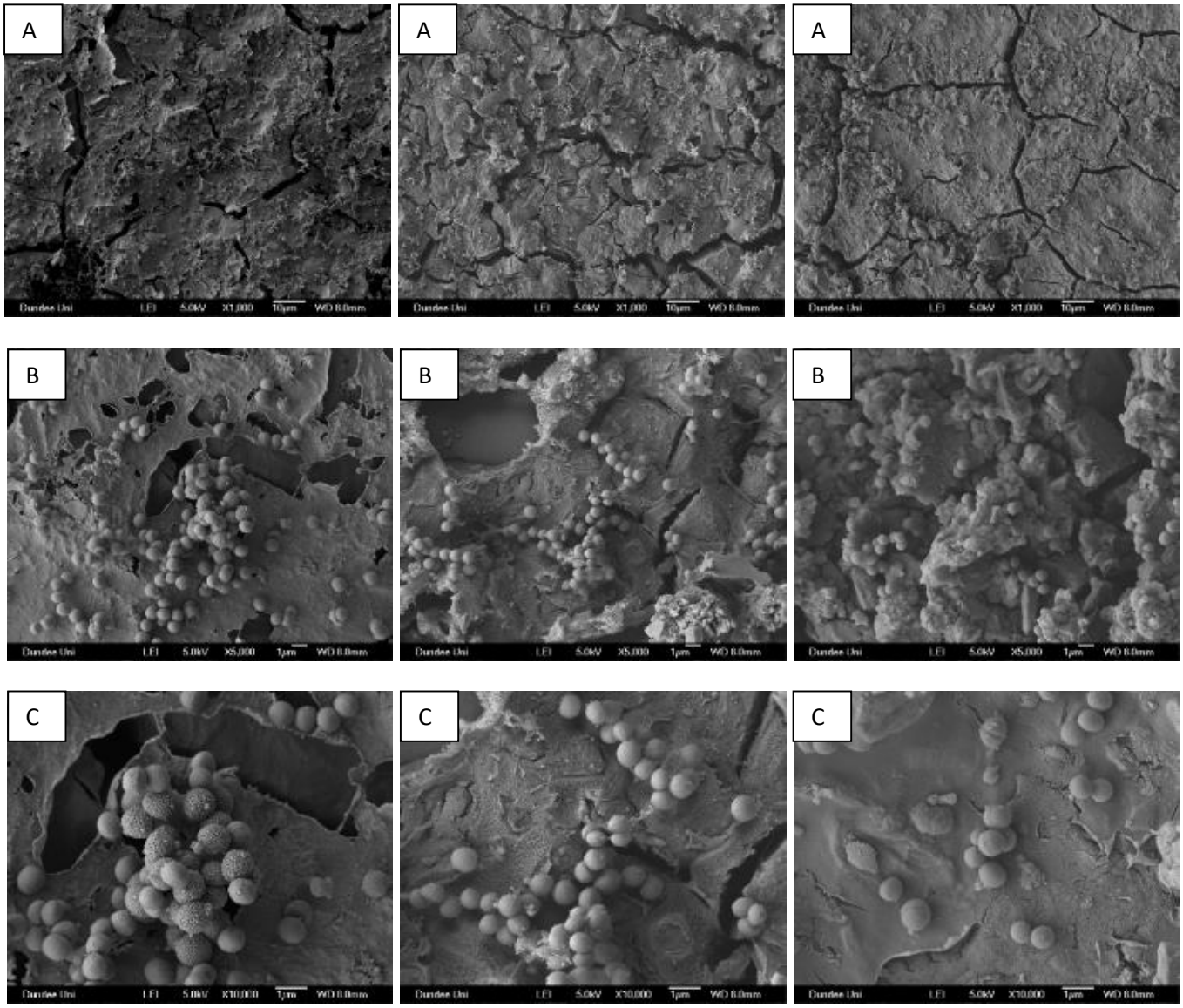


Figure 6.10 SEM images for the Intimately attached biofilm to silver modified and non-modified GIC after 24Hrs incubation under aerobic conditions at three magnifications; (A) 1000X, (B) 5000X and (C) 10000X. Showing reduced colonisation on silver modified disks compared to non-modified.

Non-modified GIC	5mg/ml Silver modified GIC	10mg/ml Silver modified GIC

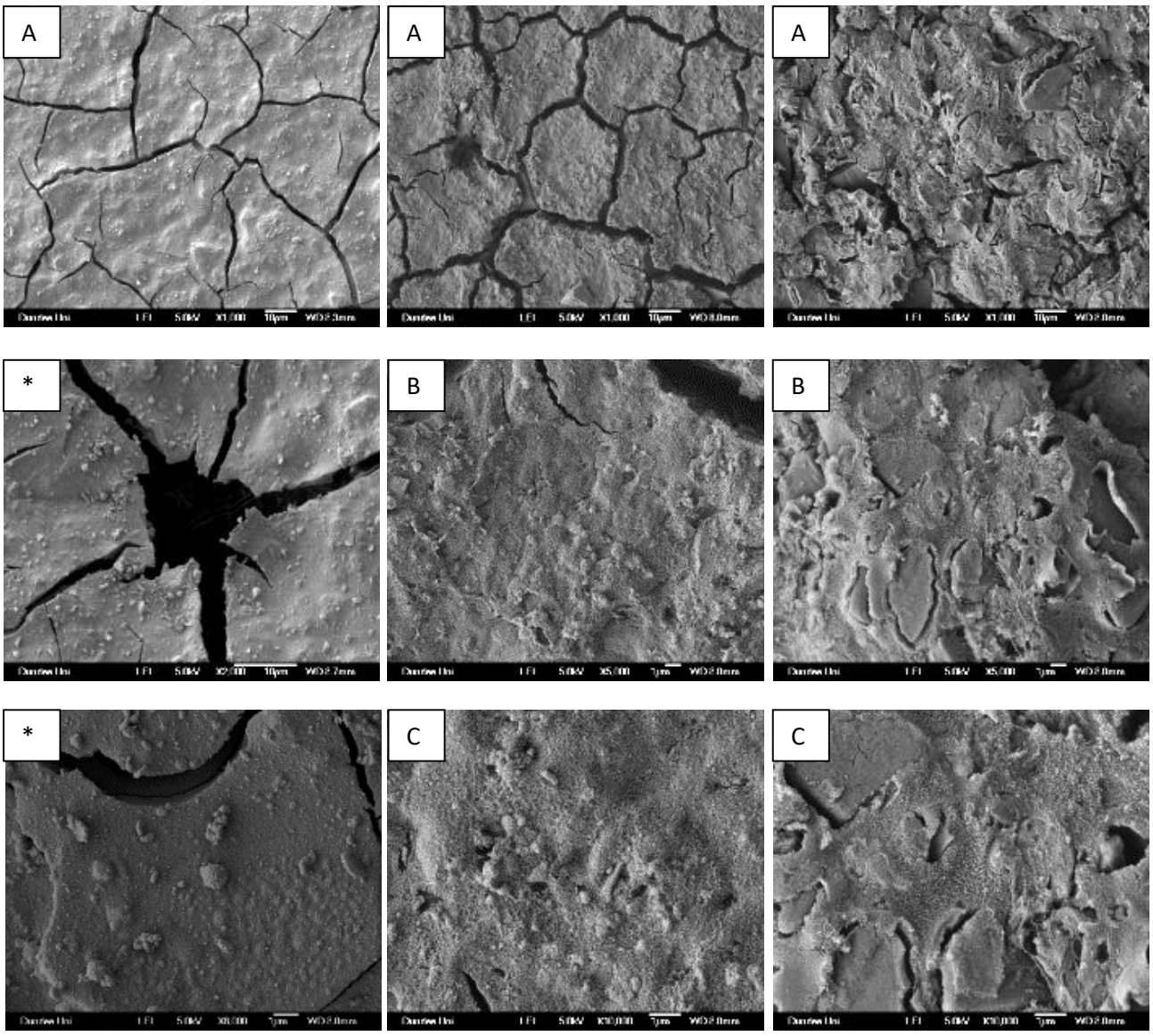


Figure 6.11 SEM images for silver modified and non-modified GIC surfaces after sonication and vortexing method used to detach the biofilm, barely showing any bacteria at three magnifications; (A) 1000X, (B) 5000X and (C) 10000X.

More debris were retained attached to silver modified disks than non-modified. Images labelled with (*) were at 2000X and 8000X, they are included to show bacterial free cavities and surfaces.

6.2.3 Discussion

Embedding antimicrobial ions in the dental materials is considered the most common strategy used to enhance anti-biofilm properties. Typical examples include fluoride then chlorhexidine (CHX), quaternary ammonium compounds etc…(Wang et al., 2014)

Numerous antibacterial agents have been incorporated into GIC to improve its antibacterial properties with a focus on the release approach, e.g. zinc ions (Osinaga et al., 2003), silver ions (Yamamoto et al., 1996), furanone which is heterocyclic organic compound known also as γ-crotonolactone (GCL) (Weng et al., 2012), and iodine and CHX (Takahashi et al., 2006).

Silver has been used for diverse dental applications ranging from silver based restorative material (Neelakantan et al., 2012) to silver coated implants (De Giglio et al., 2013), but as mentioned before Ketec Silver® is the only GIC variant which contains silver but it was added to improve its physical properties (for posterior restorations) rather than enhancing its antibacterial activity (Kilpatrick et al., 1995). The antibacterial mechanism of action of silver ions is not fully understood as stated before in section (2.5.). It can be released from dental materials in aqueous solution and can attach to the bacterial membrane and penetrate biofilm, which causes bacterial inactivation and prevents bacterial replication by binding to microbial DNA and to the sulfhydryl groups of the metabolic enzymes in the bacterial electron transport chain (Darouiche, 1999, Liao et al., 2010).

By adding the three silver solutions to glass ionomer powder, the resulting colour after fully setting is presented in Figure 6.5. It was brownish rather than black as expected from silver constituent. The colour saturation was dependent on the silver concentration; 13mg/ml is the darkest then 10mg/ml the lightest is 5mg/ml compared to normal GIC

disks. Compared to black Ketec Silver GIC, silver modified GIC disks giving better aesthetic.

After having a relatively stable silver solutions and their antibacterial activity was tested using MIC/MBC, and after having a consistent and reproducible early coloniser model, it was the step to test their adding effect on the colonisation of those three species aerobically within 24Hrs period of time and compare it to non-modified GIC.

Three outputs from each well, Floating CFUs which represent the planktonic growth around disks which will test the effect of any released ions. Wash CFUs which represent the bacterial counts assayed from washing the surfaces of the wells in other words testing what was loosely attached to the wells surfaces in which the additives may affect their colonisation by reducing or increasing their count. The last output tests what is Intimately attached to the disk surfaces after 5 times serial washes (Intimately attached CFUs), which will determine the direct effect of silver ions addition embedded in GIC.

For each CFUs assay two agar plates were used to assay each output, first is CB plates which gives the total colony count and *N. subflava* which has a distinctive colony shape, the other is MSA plates which is selective for streptococci and *S. mutans* selective because it gives a distinctive yellow halo. So, two graphs for each CFUs output.

After 24 Hrs aerobic incubation, 13mg/ml silver modified GIC disks had expanded, they were very soft and couldn't be carried with tweezer (Figure 6.6), that is may be because of high acidity content of 13 mg/ml silver which contains 2.7M citric acid. The acid content may affect the setting reaction between glass and polyacids of GIC which compromise the strength of the resultant silver modified GIC, despite that other studies found that addition of 10% citric acid by mass to GIC accelerated the setting reaction (Crisp et al., 1979) with suggested mechanism that it may have something to do with its

ability to prevent the precipitation of aluminium salts, by chelating $Al3^{+}$ ions and keeping them in solution (Lewis and McConchie, 1994).

By this mechanism, it may prevent the premature formation of ionic crosslinks involving Al^{3+} (Nicholson et al., 1988). But 2.7M citric acid seems to have an adverse effect on the setting reaction, because it might react more readily with the glass than poly (acrylic acid). Same as tartaric acid mechanism of action, but opposite to tartaric acid it retarded the rate at which aluminum polyacrylate is formed within the cement ending in suppressing the initial setting process. To confirm, citric acid solution at same molarity was added to GIC powder in the same volume as silver solution and they were incubated 24Hrs in the model and the same expansion resulted.

Although there was a decrease in the total means of bacterial growth around disks, it was not statistically significant (Friedman's two-way ANOVA by rank test), bacterial counts around non-modified GIC disks was the least in total 2.72×10^{8} CFUs/ml compared to control wells without any disks in 5.54×10^{8} CFUs/ml, 3.05×10^{8} and 4.1×10^{8} CFUs/ml were 10mg/ml and 5mg/ml counts (Figure 6.7).

N. subflava also was reduced in wells with either modified or non-modified GIC, the least were associated with silver modified disks (Figure 6.7) and again was not statistically significant (Appendix 2 section 10.2.1 for further statistical analysis details).

S. oralis was the main species retrieved from the wells, compared to control wells the number of *S. oralis* was less in wells containing disks with no significant difference between them (Figure 6.7).

S. mutans counts in wells with silver modified GIC were significantly lower than control wells ($P < 0.05$ Friedman's two-way ANOVA by rank test) the lowest was in well containing 10mg/ml silver modified GIC disks 2.97×10^{7} CFUs/ml on average. But in

wells with non-modified GIC there was no significant difference (Friedman's two-way ANOVA by rank test) (Table 6.3).

However, with the Wash assay there was significant reduction in the total bacterial count from wells with silver modified disks compared to control wells rather than non-modified GIC (Figure 6.8) (Table 6.4). But with *N. subflava* there was no significant reduction. That was not the case with what has been assayed from MSA plates, because neither *S. oralis* nor *S. mutans* has any significant reduction. This indicated that the presence of modified discs has a negligible effect on the bacterial counts loosely attached to the wells (Figure 6.8) (Table 6.5).

Colonisation on GIC disks has significantly reduced in total by addition of silver solutions, it can be seen in (Figure 4.9). *S. oralis* CFUs was significantly reduced on disks modified with 10mg/ml silver solution but surprisingly *S. mutans* count was higher than both 5mg/ml silver modified and non-modified GIC (Figure 6.9) (Table 6.6), and (6.7).

SEM images revealed a decrease in colonisation on both silver modified disks compares to non-modified (Figure 6.10), in addition most of the bacterial community on disks were successfully detached by sonication and vortexing leaving behind some debris, which are most probably bacterial products dead cells and extracellular matrix. More debris were on silver modified surfaces rather than non-modified which may suggest more killing activity by direct contact with silver ions on disk surfaces. Also PVA might be involved in this finding as debris associated with 10mg/ml silver modified disks which has PVA in its formulation showed more gross debris than 5 mg/ml silver modified GIC (Figure 6.11).

From all the previous findings it could be concluded that silver antibacterial activity works by leaching out from disks into media but their effect was not significant, it can be confirmed by measuring the release of silver in DMM using an electrode which will be in the further studies of this project. Fluoride also seems to have an effect comparable to silver but it was not significant and this finding goes with the clinical finding around the debatable effect of fluoride release from GIC as it might be diluted and buffered with saliva.

Aerobically within 24Hrs period of time silver additives enhanced the antibacterial activity of GIC by reducing colonisation especially *S. oralis* which is a main early coloniser.

6.3 The Effect of Silver Additives on the Attachment and Survival of Early Coloniser Model under Anaerobic Conditions for 24 Hours

The supra-gingival plaque community is considered to be quite distinct from sub-gingival plaque. The dominant inhabitant of Supra-gingival plaque are Gram-positive facultative bacteria, especially streptococci and actinomyces, although a wide variety of organisms including organisms classified as obligate aerobes are present, including species of the genera Neisseria. Sub-gingival plaque is also classically considered to contain large numbers of Gram-positive bacteria including different genera. However, it's the increase in Gram-negative, anaerobic bacteria, and wider species diversity that characterises these microbial communities (Ximenez-Fyvie et al., 2000). Supra-gingival plaque can be further subdivided into the plaque on smooth surfaces that are freely accessible to saliva and to oxygen of the air, plaque at contact points of the teeth, which are less freely accessible and plaque in pits and fissures, which is relatively inaccessible. The latter plaque may be enriched in anaerobic species and in acid-tolerant bacteria such as *S. mutans* and Lactobacillus and (Marquis, 1995).

Consequently, dental plaque ecology and metabolism is different from site to site, depending on the presence of oxygen and the mix of nutrients available. In addition, oral microorganisms are highly adaptable, many being facultative anaerobes, and therefore dental plaque organisms may behave differently under anaerobic conditions or acid challenge (Daniluk et al., 2006).

To test how early coloniser model species, behave under anaerobic conditions in the presence of silver modified and non-modified GIC disks, the following experiment was conducted.

6.3.1 Materials and Methods

All the same as in the previous experiment (6.2.1) except the plates were incubated anaerobically for 24Hrs at 37ºC.

6.3.2 Results

Figure 6.12-6.14 demonstrate the three outputs from wells using CFUs assay. Similarly, to the previous experiment. The planktonic growth around disks (Floating), what was loosely attached to the well surfaces (Wash) and what remained attached to disk surfaces after 5 times serial washes (Intimately attached).

Intimately attached biofilm SEM images, and detachment was confirmed by SEM in (Figure 6.15).

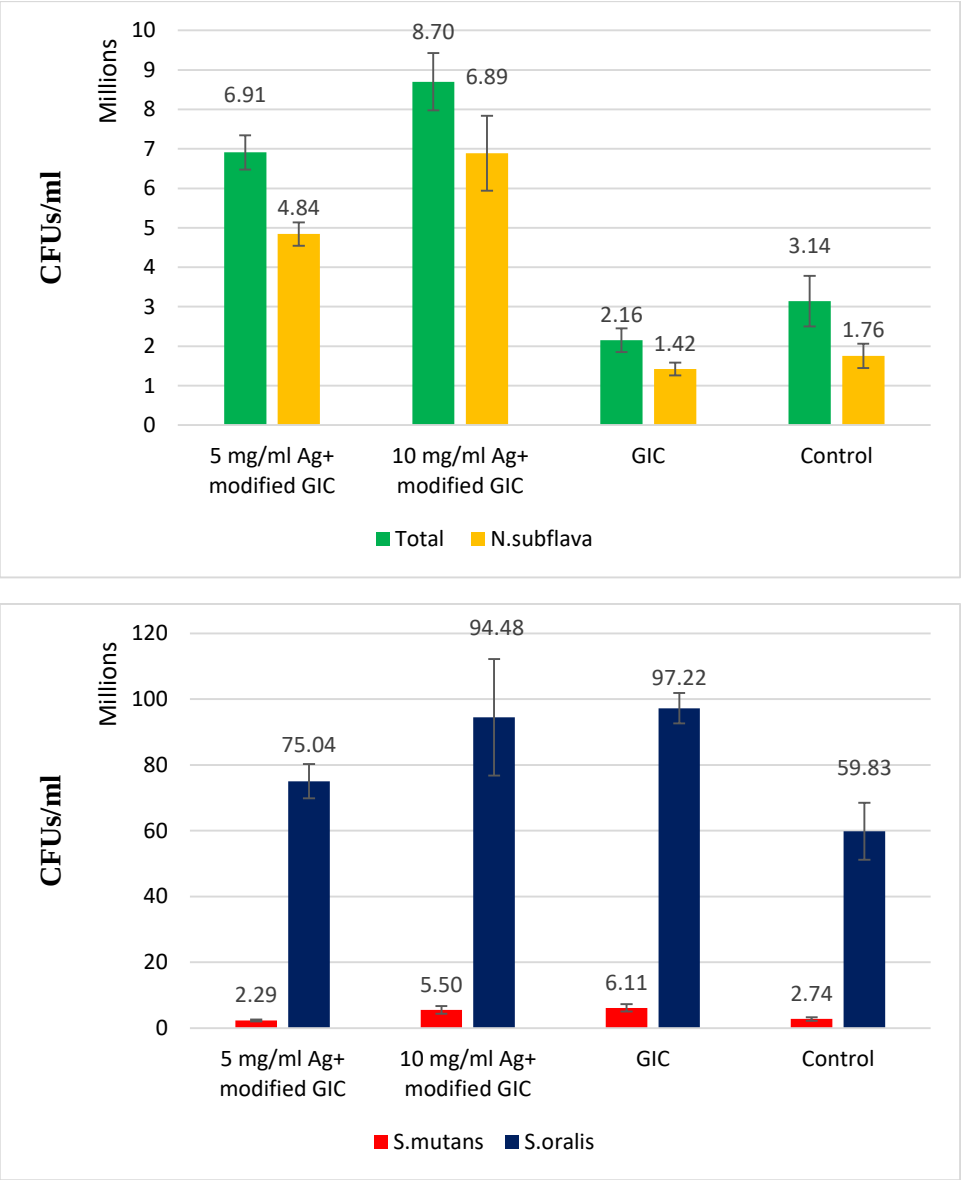


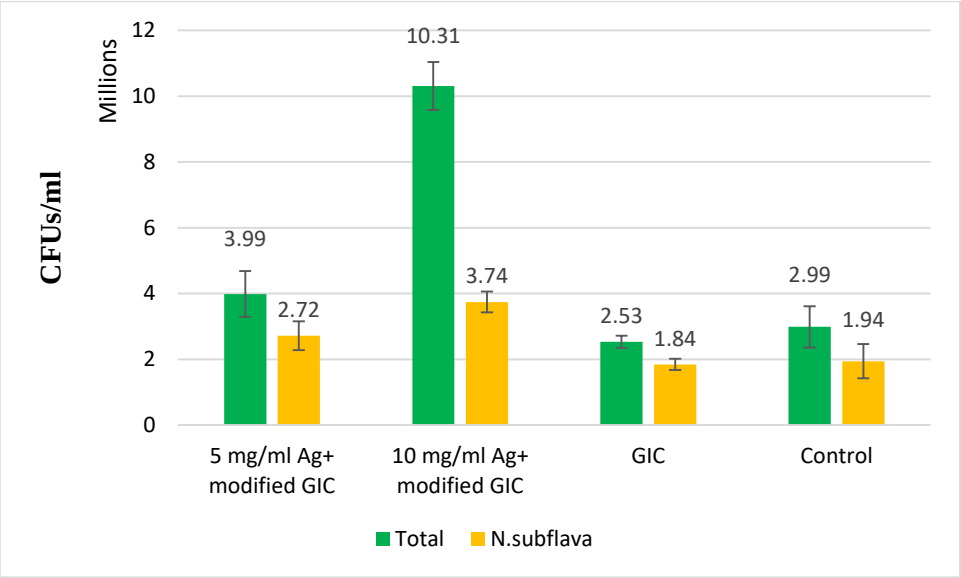
Figure 6.12 Graphs represent viable bacterial count (CFUs) (Mean ± SEM *n* =9) in millions for the planktonic bacterial growth around disks (Floating) in Total and for each of the three species after anaerobic incubation for 24 Hrs. *N. subflava* was dominating rather than *S. oralis*, Total CFUs and *N. subflava* CFUs was significantly higher in wells with silver modified GIC compared to both control and to wells with non-modified GIC (Friedman's two-way ANOVA by rank test) ($p < 0.05$).

Table 6.8 Pairwise comparison of the Total Floating CFUs showing a highly significant difference between wells containing 10mg/ml silver modified GIC in comparison to non-modified GIC, 5mg/ml silver modified GIC in comparison to non-modified GIC and in wells containing 10mg/ml silver modified GIC in comparison to control wells (**p <0.05).*

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	0.865
10mg/ml silver modified GIC – ChemFil Superior GIC	***0.000****
10mg/ml silver modified GIC – Control	***0.004****
5mg/ml silver modified GIC – ChemFil Superior GIC	***0.028****
5mg/ml silver modified GIC – Control	0.331
ChemFil Superior GIC - Control	1.000

Table 6.9 Pairwise comparison of the *N. subflava* Floating CFUs showing a highly significant difference between all wells except 10mg/ml silver modified GIC in comparison to 5mg/ml silver modified GIC, and in wells containing non-modified GIC in comparison to control wells(**p <0.05).*

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	1.000
10mg/ml silver modified GIC – ChemFil Superior GIC	***0.001****
10mg/ml silver modified GIC – Control	***0.001****
5mg/ml silver modified GIC – ChemFil Superior GIC	***0.028****
5mg/ml silver modified GIC – Control	***0.049****
ChemFil Superior GIC - Control	1.000



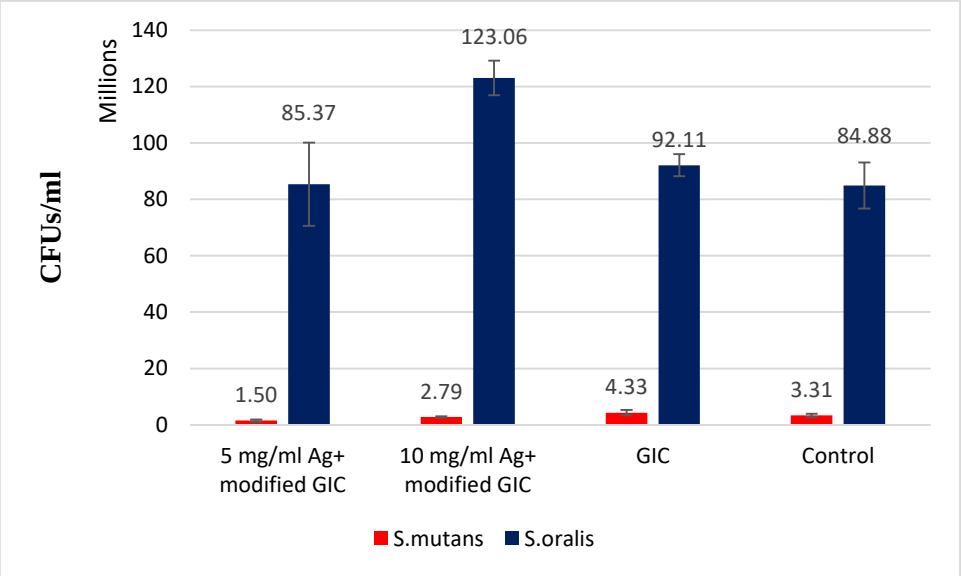


Figure 6.13 Graphs represent viable bacterial count (CFUs) (Mean ± SEM *n* =9) in millions in Total and for the three species assayed from wells surfaces washing (Wash) after anaerobic incubation for 24 Hrs. *N. subflava* was dominating rather than *S. oralis* in control, 5mg/ml modified and non-modified GIC wells. Total CFUs and *N. subflava* CFUs was significantly higher in wells with 10mg/ml silver modified GIC compared to both control and to wells with non-modified GIC. *S. oralis* Wash CFUs significantly higher in wells contains 10mg/ml silver modified GIC compared to non-modified. *S. mutans* Wash CFUs significantly higher in wells contains 10mg/ml silver modified GIC compared to 5mg/ml modified (Friedman's two-way ANOVA by rank test) ($p < 0.05$).

Table 6.10 Pairwise comparison of the Total Wash CFUs showing a highly significant difference between wells containing 10mg/ml silver modified GIC in comparison to non-modified GIC, 10mg/ml silver modified GIC in comparison to control (* *p <0.05).*

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	0.106
10mg/ml silver modified GIC – ChemFil Superior GIC	***0.002****
10mg/ml silver modified GIC – Control	***0.001****
5mg/ml silver modified GIC – ChemFil Superior GIC	1.000
5mg/ml silver modified GIC – Control	0.865
ChemFil Superior GIC - Control	1.000

Table 6.11 Pairwise comparison of the *N. subflava* Wash CFUs showing a highly significant difference between wells containing 10mg/ml silver modified GIC in comparison to non-modified GIC, 10mg/ml silver modified GIC in comparison to control (* *p <0.05).*

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	0.602
10mg/ml silver modified GIC – ChemFil Superior GIC	***0.011****
10mg/ml silver modified GIC – Control	***0.006****
5mg/ml silver modified GIC – ChemFil Superior GIC	0.865
5mg/ml silver modified GIC – Control	0.602
ChemFil Superior GIC - Control	1.000

Table 6.12 Pairwise comparison of the *S. oralis* Wash CFUs showing a highly significant difference between wells containing 10mg/ml silver modified GIC in comparison to non-modified GIC (* *p <0.05)*.

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	0.106
10mg/ml silver modified GIC – ChemFil Superior GIC	***0.037****
10mg/ml silver modified GIC – Control	0.064
5mg/ml silver modified GIC – ChemFil Superior GIC	1.000
5mg/ml silver modified GIC – Control	1.000
ChemFil Superior GIC - Control	1.000

Table 6.13 Pairwise comparison of the *S. mutans* Wash CFUs showing a highly significant difference between wells containing 10mg/ml silver modified GIC in comparison to 5mg/ml silver modified GIC (* *p <0.05)*.

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	***0.028****
10mg/ml silver modified GIC – ChemFil Superior GIC	1.000
10mg/ml silver modified GIC – Control	1.000
5mg/ml silver modified GIC – ChemFil Superior GIC	0.215
5mg/ml silver modified GIC – Control	0.106
ChemFil Superior GIC - Control	1.000

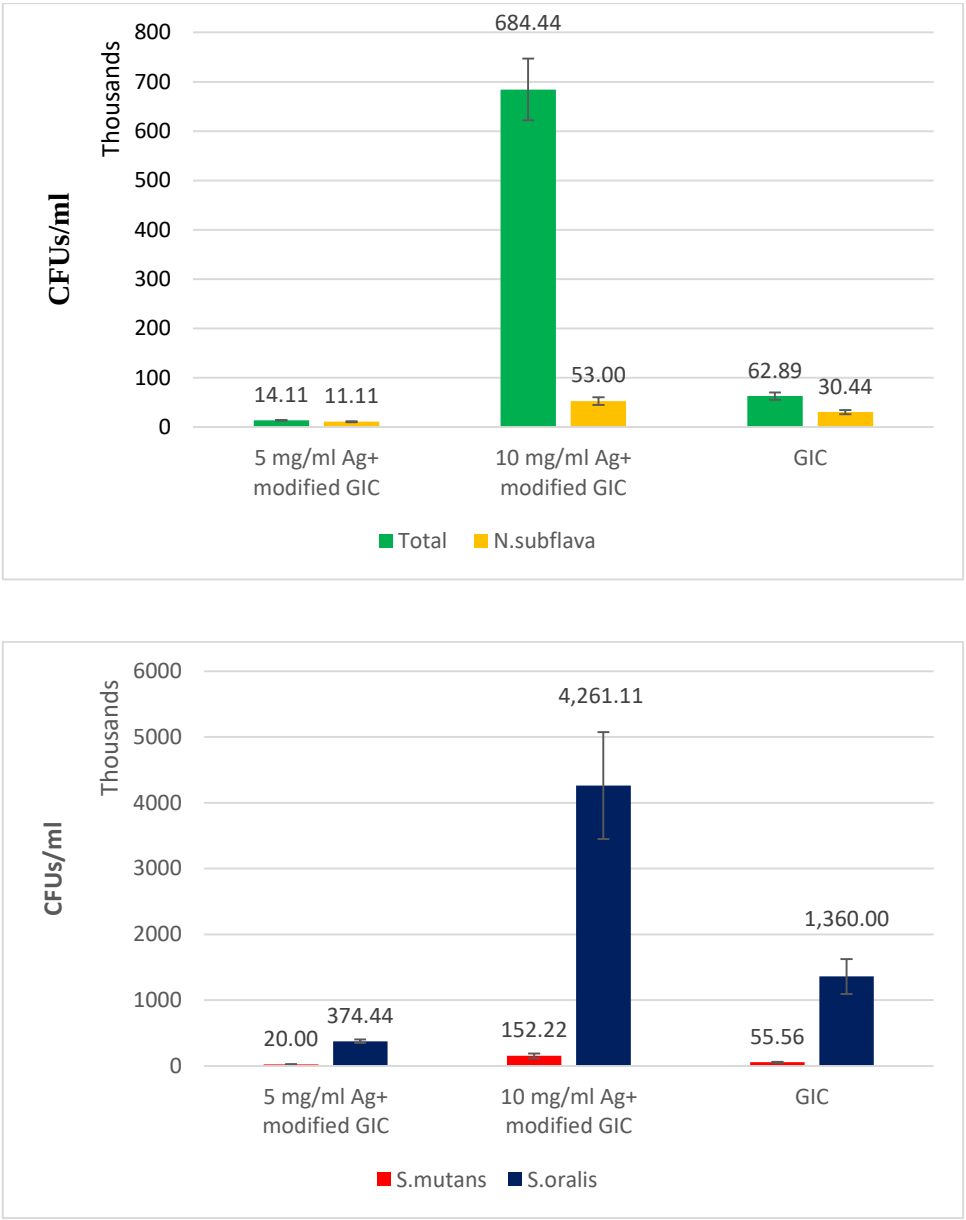


Figure 6.14 Graphs represent viable bacterial count (CFUs) (Mean ± SEM *n* =9) in thousands in Total and for the three species assayed from disk surfaces (Intimately Attached) after anaerobic incubation for 24 Hrs. *N. subflava* was dominating rather than *S. oralis* in 5mg/ml modified and non-modified GIC wells. Total CFUs and each species CFUs was significantly higher in wells with 10mg/ml silver modified GIC compared to 5mg/ml silver modified GIC but not to non-modified GIC (Friedman's two-way ANOVA by rank test) ($p < 0.05$).

Table 6.14 Pairwise comparison of the Total Intimately Attached CFUs showing a highly significant difference between counts from 10mg/ml silver modified GIC in comparison to 5mg/ml silver modified GIC (* *p <0.05)*. Same results with analysis of *N. subflava* and *S. oralis* CFUs.

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified	***0.000****
10mg/ml silver modified GIC – ChemFil Superior GIC	0.102
5mg/ml silver modified GIC – ChemFil Superior GIC	0.102

Table 6.15 Pairwise comparison of the *S. oralis* Intimately Attached CFUs showing a highly significant difference between wells containing 10mg/ml silver modified GIC in comparison to non-modified GIC (* *p <0.05)*.

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified	***0.000****
10mg/ml silver modified GIC – ChemFil Superior GIC	0.102
5mg/ml silver modified GIC – ChemFil Superior GIC	0.231

Non-modified GIC	5mg/ml Silver modified GIC	10mg/ml Silver modified GIC

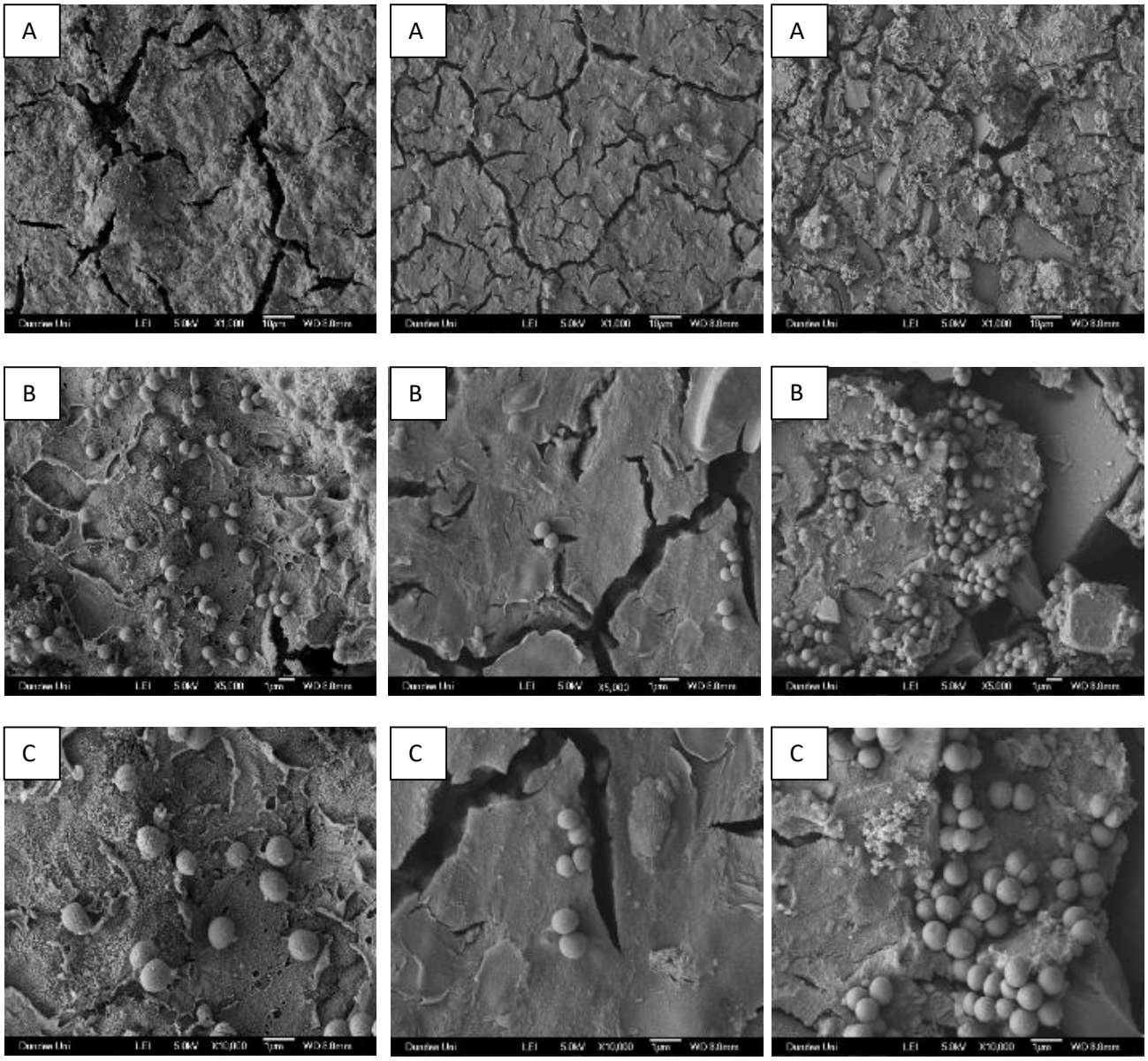


Figure 6.15 SEM images for the Intimately attached biofilm to silver modified and non-modified GIC after 24Hrs incubation under anaerobic conditions at three magnifications; (A) 1000X, (B) 5000X and (C) 10000X.

Showing reduced colonisation on 5mg/ml silver modified disks and increased colonisation on 10mg/ml silver modified disks compared to non-modified disks.

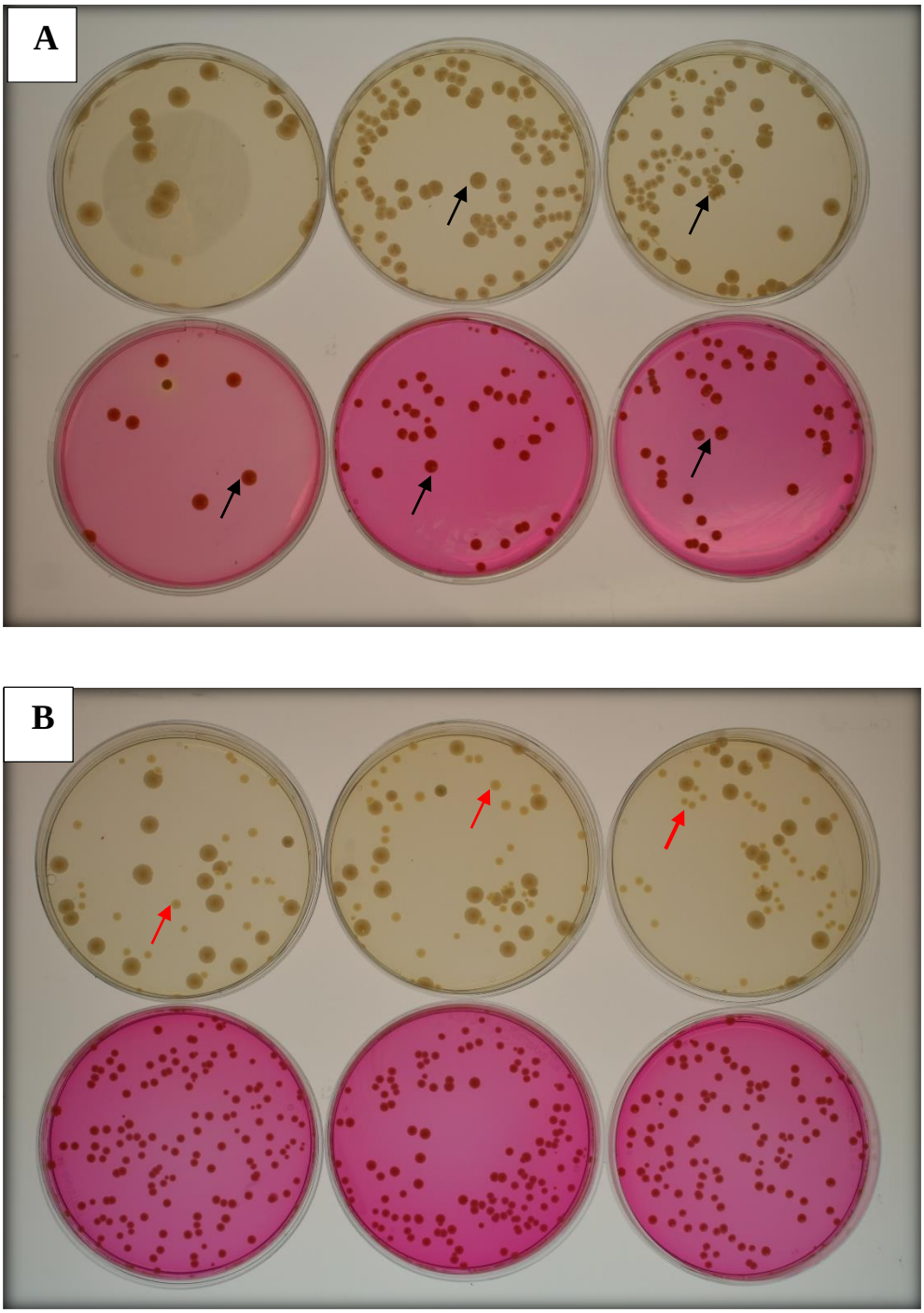


Figure 6.16 CFUs assay after 24Hrs incubation Aerobically (A) and anaerobically (B). Presenting the community shift and domination from *S. oralis* (Black arrows) aerobically to *N. subflava* (Red arrows) anaerobically, differentiation was depending on colony morphology.

6.3.3 Discussion

Bacteria can be divided into two categories; aerobic and anaerobic bacteria, depending on the respiration type pathway i.e. the presence of oxygen. Both aerobic and anaerobic respiration involves a first stage called glycolysis which is the metabolic pathway that convert glucose to pyruvate. The additional stage in anaerobic is fermentation in which the pyruvate undergoes a simple redox reaction ending with lactic acid formation, however in aerobic conditions there is the Krebs cycle and electron transfer chain which convert pyruvate to acetyl-coA then it enters the kerbs cycle to produce NADH and FADH which are used by electron transport chain to form ATP energy molecules by electron reduction. Finally, combustion is complete in aerobic respiration, and incomplete during anaerobic respiration (Daniluk et al., 2006).

Aerobic respiration takes place in the presence of oxygen, produces a large amount of energy with Carbon dioxide and water are by-products. While anaerobic respiration takes place without the use of oxygen, produces small amounts of energy and alcohol or lactic acid or other compounds as by-products depending on the kind of cells that are active (Scott, 2005). Under anaerobic conditions oxygen cannot act as an acceptor molecule allowing protons to be excluded and intermediate proteins to be regenerated.

Aerobic bacteria are the bacteria that use dissolved oxygen for their metabolic reactions. They may exist as obligate aerobes like *N. subflava*, which grow only in the presence of oxygen, or exist as facultative anaerobes like *S. oralis* and *S. mutans* , which grow in the presence of oxygen, but can tolerate anaerobic conditions, as well. The ultimate hydrogen acceptor of aerobes is oxygen, which they use to reduce energy source and produce carbon dioxide and water as end products (Daniluk et al., 2006).

Bacteria that do not need dissolved oxygen for their metabolisms are called anaerobes. They basically use the oxygen in chemical compounds for their metabolic reactions. Unlike aerobes, anaerobic bacteria cannot use molecular oxygen as terminal electron acceptors; instead, they use nitrate, sulphate, carbon dioxide, and organic compounds as terminal acceptors (Marquis, 1995).

So in summery the main differences between aerobic and anaerobic bacteria are;

- Catalase, the enzyme which splits hydrogen peroxide is found in most aerobes but is absent in anaerobes.
- Aerobes can completely reduce carbon energy source to water and carbon dioxide using oxygen, whereas anaerobes use nitrates and sulphates instead of oxygen, hence producing gasses such as sulphur dioxides, methane, ammonia etc.
- Unlike aerobes, anaerobes do not obtain much energy per unit of substrate they metabolised.

As a results of anaerobic incubation of the model species with silver modified and non-modified disks, biofilm formed was different from the biofilm formed under aerobic conditions. First, the growth rate was slower anaerobically for example after 24Hrs floating CFUs in total aerobically was equal to 5.54×10^{8} while it was 3.14×10^{6} in anaerobic conditions. Second, *N. subflava* is an obligate aerobic bacterium, however it was retrieved from the three outputs (Floating, Wash and Intimately attached assay), but it didn't grow either as single species in MIC/MBC experiment 6.1, or as single cultures without additives. The surprising growth of *N. subflava* anaerobically may be explained by the recently described entero-salivary nitrate-nitrite-nitric oxide pathway which provides bioactive NO from dietary nitrate sources. Interestingly, this pathway is dependent upon oral nitrate-reducing bacteria, *N. subflava* contained a nitrous oxide reductase (Nos) enzyme which is part of the enzymes involved in the pathway that enable

the commensal Neisseria species to grow under anaerobic conditions, produce and degrade NO, and to establish an NO Steady state concentration during entero-salivary nitrate-nitrite-nitric oxide pathway (Hyde et al., 2014, Barth et al., 2009). Third, the percentage of *N. subflava* CFUs from Total CFUs was higher than it was under aerobic conditions (Table 6.16) and (Figure 6.12) which indicates that *N. subflava* was the main coloniser (Figure 6.16).

Table 6.16 *N. subflava* percentage from Total bacterial count (CFUs) aerobically compared to anaerobic conditions.

Mean *N. subflava* % from total CFUs			
	Floating output	Wash output	Intimately attached
Aerobic condition	18%	13.25%	3.93%
Anaerobic condition	67.75%	60.25%	44.56%

Regarding the effect of silver additives on the colonisation and behaviour of species model, from floating assay, wells contained silver modified GIC exhibit higher growth in total and in *N. subflava* compared to control wells and wells with non-modified GIC. (Figure 6.12), the growth was significantly different between specific wells as shown in (Table 6.8 and 6.9) after Friedman's two-way ANOVA by rank test been conducted. Also *S. oralis* counts were more than *S. mutans* in all wells and there was no significant difference between the counts of *S. oralis* neither *S. mutans* statistically using Friedman's two-way ANOVA by rank test.

The Wash assay from well surfaces containing 10 mg/ml silver modified GIC was significantly higher in total mean and *N. subflava* total mean, compared to control wells and wells with non-modified GIC (Figure 6.13), (Table 6.10 and 6.11). While from wells with 5mg/ml silver modified GIC the counts were slightly higher but were not significant. *S. oralis* counts were significantly higher also from wells with 10mg/ml silver modified GIC than wells with non-modified GIC (Figure 6.13), (Table 6.12), and also the same case was for *S. mutans* counts but it was less in wells with 10mg/ml (Table 6.13).

10mg/ml silver modified GIC disks promoted colonisation of early coloniser model significantly by 91% in total from non-modified GIC disks, while 5mg/ml decreased the colonisation in total by 77.5%. It was also the same scenario in the *S. oralis* assay where 10mg/ml silver modified GIC disks increase their colonisation by 68%, however 5mg/ml decrease *S. oralis* colonisation by 72.5%, (Figure 6.14) , (Tables 6.14 and 6.15), this was opposite to the behaviour of the biofilm aerobically where 10 mg/ml silver modified GIC decreased the colonisation by 64.6% in total and 53% in S. oralis counts. This finding led us to postulate that if the materials show antibacterial activity aerobically, it may not the case under anaerobic conditions and that was confirmed by SEM images in (Figure 6.15) where 10mg/ml silver addition to GIC enhanced bacterial colonisation anaerobically compared to non-modified GIC. However, 5mg/ml silver addition decreased the colonisation compared to non-modified GIC. In addition, sonication and vortexing successfully detached anaerobic biofilms from disk surfaces.

6.4 The Effect of Silver Additives on Viability of Early Coloniser Model under Aerobic and Anaerobic Conditions for 24 Hours Using MTT Assay

The widely used gold standard method in determining the bacterial cell count is Colony Forming Units (CFUs) (Hazan et al., 2012). The CFU method has two important advantages, first the capacity for counts of any number of bacteria using dilutions, if too many, or concentrations if too few. Second, only viable bacteria are counted with this method as the CFU method excludes dead bacteria and debris. The most important disadvantage of the CFU method is that clumps of bacteria cells can be miscounted as single colonies. In addition, CFU results are usually obtained after 1–3 days, which makes the method unsuitable for serial longitudinal studies. Above that the CFU method is also relatively time-consuming and quite tedious, so the need for another assay is inevitable like MTT assay to cover some of the limitations of CFUs assay.

6.4.1 Materials and Methods

6.4.1.1 Bacterial Species; *S. mutans* , *S. oralis* and *N. subflava*.

6.4.1.2 Growth Medium; CB and DMM.

6.4.1.3 Solutions

- Additives: silver solutions; 5mg/ml and 10mg/ml.
- DMSO (Dimethyl sulphoxide) (SIGMA # D-5859, Sigma-Aldrich Company Ltd. Dorset, England).
- MTT (3-(4, Dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) (SIGMA # M-2128, Sigma-Aldrich Company Ltd. Dorset, England).
- PBS (Phosphate buffered saline) (SIGMA # P-4417, Sigma-Aldrich Company Ltd. Dorset, England).

6.4.1.4 Specimens

24 disks in total were prepared as in section (4.1.3.1), 8 non-modified ChemFil Superior disks, and 16 silver modified ChemFil Superior disks 8 for each silver concentration.

6.4.1.5 Culturing Methods and Growth Conditions

The same culturing and growth conditions were used as in section (5.9.1.3), except in the arrangement of modified and non-modified disks through the wells; for each experiment 24 disks were tested, 8 for each silver solution and 8 non-modified GIC. 4 wells left as control contained the early coloniser species without any disk in. Half of each state specimens were incubated aerobically at 37ºC and the other half were under anaerobic conditions and both for 24Hrs.

The planktonic growth around disks (Floating), what was loosely attached to wells surfaces (Wash) and what remained attached to disk surfaces after 5 times serial washes (Intimately attached) was assayed by taking 100µl from the culture around the disks and 100µl from the washed PBS to 24 well plate and follow the same protocol in section (4.3.2). Each experiment was repeated 3 times.

Absorbance values obtained for each well represent the amount of MTT reduction, which is proportional to the number of viable cells. In order to assess the percentage of viable cells present in each well, the absorbance values were related to those of the control. This was achieved by setting the mean absorbance of the control at 100%.

The effects of silver additive to ChemFil Superior GIC were compared to each other using the percent viability values as indicators of bacterial viability, and the data were analysed to determine the effect of type of materials on cell viability. The data was analysed by Friedman's two-way ANOVA by rank test ($P < 0.05$) being taken as significant.

6.4.2 Results

The average viability percentages for each output aerobically and anaerobically with their correspondence statistical analysis results are in the (Figures 6.17 and 6.18) and (Tables 6.17-6.22).

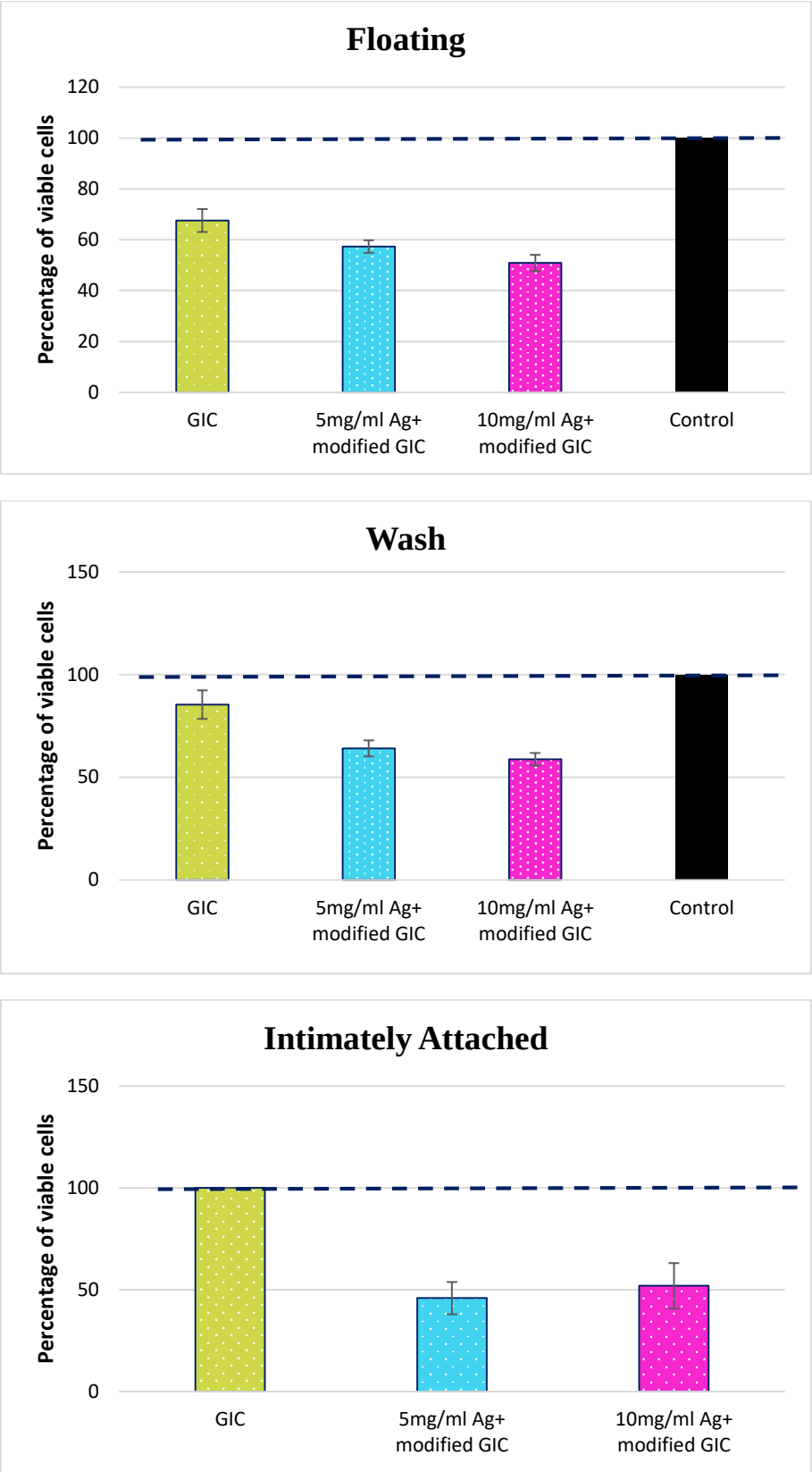


Figure 6.17 Percentage of Total bacterial cell viability (Mean ± SEM *n* =12) relative to the control (100% viability) for materials after 24Hrs aerobic incubation.

Table 6.17 Pairwise comparison of the aerobic Floating MTT viability percentages showing a highly significant difference between wells containing 10mg/ml silver modified GIC in comparison to non-modified GIC, 5mg/ml silver modified GIC in comparison to control and non-modified GIC in comparison to control *(*P < 0.05).*

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	1.000
10mg/ml silver modified GIC – ChemFil Superior GIC	0.581
10mg/ml silver modified GIC – Control	***0.000****
5mg/ml silver modified GIC – ChemFil Superior GIC	1.000
5mg/ml silver modified GIC – Control	***0.000****
ChemFil Superior GIC - Control	***0.027****

Table 6.18 Pairwise comparison of the aerobic Wash MTT viability percentages showing a highly significant difference between wells containing 10mg/ml silver modified GIC in comparison to non-modified GIC and control wells. 5mg/ml silver modified GIC in comparison to control (**P < 0.05).*

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	1.000
10mg/ml silver modified GIC – ChemFil Superior GIC	***0.016****
10mg/ml silver modified GIC – Control	***0.000****
5mg/ml silver modified GIC – ChemFil Superior GIC	0.347
5mg/ml silver modified GIC – Control	***0.016****
ChemFil Superior GIC - Control	1.000

Table 6.19 Pairwise comparison of the Intimately Attached MTT viability percentages aerobically showing a highly significant difference between viability percentages of bacterial cells Intimately attached to 10mg/ml silver modified GIC in comparison to non-modified GIC. And between 5mg/ml silver modified GIC in comparison to non-modified GIC (*$P < 0.05$).

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	1.000
10mg/ml silver modified GIC – ChemFil Superior GIC	***0.002****
5mg/ml silver modified GIC – ChemFil Superior GIC	***0.003****

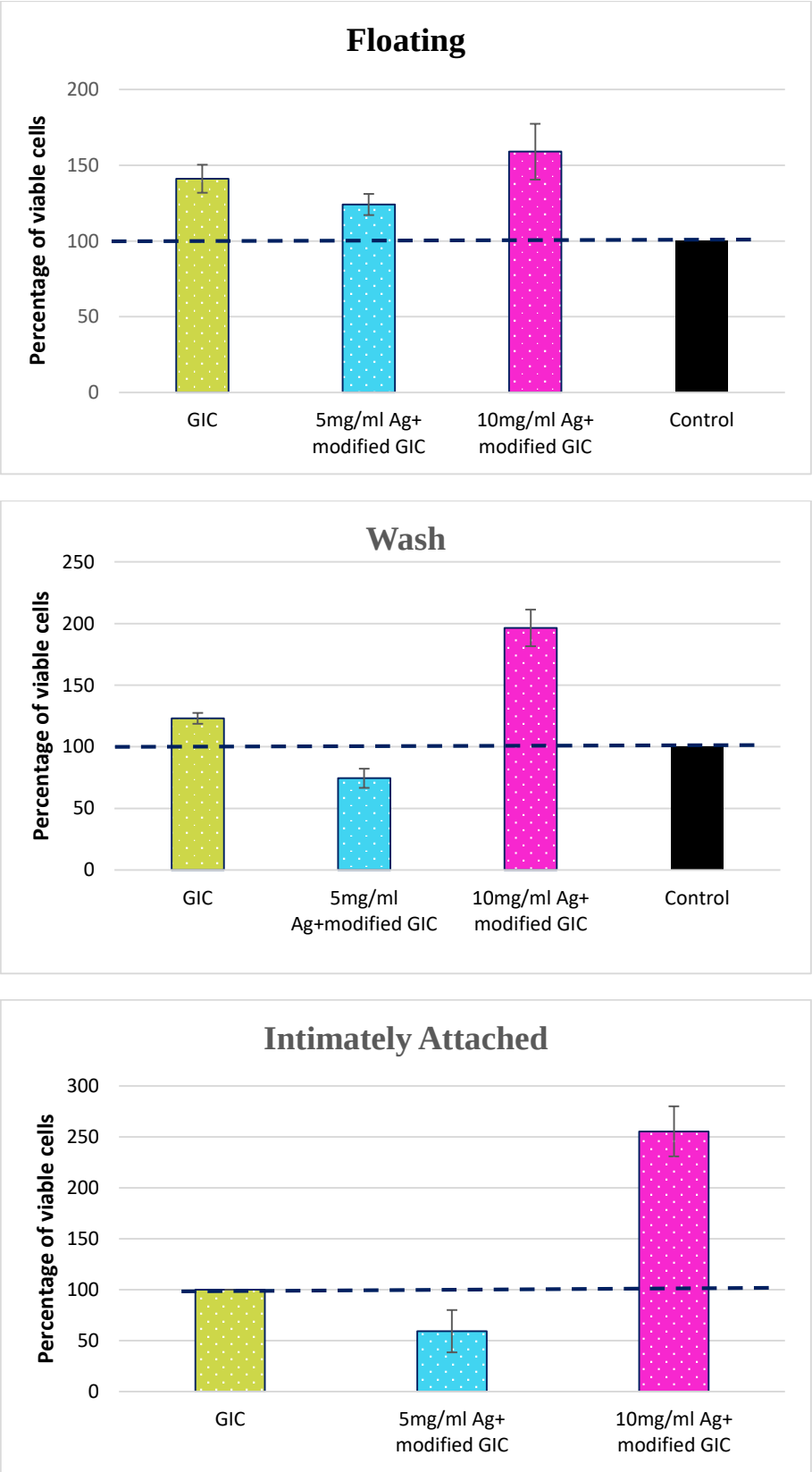


Figure 6.18 Percentage of Total bacterial cell viability (Mean ± SEM *n* =12) relative to the control (100% viability) for materials after 24Hrs anaerobic incubation.

Table 6.20 Pairwise comparison of the anaerobic Floating MTT viability percentages showing a highly significant difference between wells containing 10mg/ml silver modified GIC in comparison to control, non-modified GIC in comparison to control (*$P < 0.05$).

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	0.798
10mg/ml silver modified GIC – ChemFil Superior GIC	1.000
10mg/ml silver modified GIC – Control	***0.002****
5mg/ml silver modified GIC – ChemFil Superior GIC	0.683
5mg/ml silver modified GIC – Control	0.197
ChemFil Superior GIC - Control	***0.001****

Table 6.21 Pairwise comparison of the anaerobic Wash MTT viability percentages showing a highly significant difference between wells containing 10mg/ml silver modified GIC in comparison to 5mg/ml silver modified GIC and control wells. 5mg/ml silver modified GIC in comparison to non-modified GIC (*$P < 0.05$).

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	***0.000****
10mg/ml silver modified GIC – ChemFil Superior GIC	0.492
10mg/ml silver modified GIC – Control	***0.001****
5mg/ml silver modified GIC – ChemFil Superior GIC	***0.003****
5mg/ml silver modified GIC – Control	0.928
ChemFil Superior GIC - Control	0.239

Table 6.22 Pairwise comparison of the Intimately Attached MTT viability percentages anaerobically showing a highly significant difference between viability percentages of bacterial cells Intimately attached to 10mg/ml silver modified GIC in comparison to 5mg/ml silver modified GIC and non-modified GIC (* *P < 0.05)*.

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	***0.000****
10mg/ml silver modified GIC – ChemFil Superior GIC	***0.013****
5mg/ml silver modified GIC – ChemFil Superior GIC	0.922

6.4.3 Discussion

As mentioned in section 4.3, MTT assay is a colorimetric assay for assessing cell metabolic activity. NAD (P)H-dependent cellular oxidoreductase enzymes, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple colour (Berridge and Tan, 1993).

Consequently, the intensity of purple colour reflects the amount of cell viability indirectly; more purple colour with more bacterial cell viability. The purple colour intensity was measured by a plate reader at 550 nm wavelength (wavelength corresponds to purple colour), another reading at 620 nm was taken to make sure we got the exact colour by making calculations and subtracting 550nm reading from 620nm reading.

Wells without disks were considered control wells, the average reading from them was considered as 100% viability. By that the readings from other wells were related to the average reading and a percentage was calculated for each reading (Tables in Appendix 2 section 10.2.3).

Aerobically after 24Hrs the viability of bacterial cells in wells with modified and non-modified GIC were significantly lower than control wells. Wells with 10mg/ml silver modified GIC were the lowest on average and with non-modified GIC were the highest (Figure 6.17) (Table 6.17). This indicated that the presence of disks affects the viability of the bacterial community around them and silver modified GIC disks were more effective than non-modified GIC. Whereas the viability of bacterial cells of Wash assay from silver modified wells were also significantly lower than control wells. Wells with 10 mg/ml silver modified GIC were lower than 5 mg/ml silver modified GIC, although the Wash viability from wells with non-modified GIC was lower than the controls but it

was not statistically significant. This is also considered an indication of the effect of additives on the viability of bacterial cells loosely attached to the well surfaces (Figure 6.17) (Table 6.18). The bacterial cell viability of Intimately attached assay from silver modified disks were significantly lower than non-modified GIC. To that end silver formulation additives to GIC were effective in enhancing the antibacterial activity of GIC aerobically within the parameters of our experiment (Figure 6.17) (Table 6.19).

In anaerobic conditions the viability of Floating assay was significantly higher in wells with 10mg/ml silver modified GIC and non-modified GIC, although the viability from wells with 5mg/ml silver modified GIC were higher than the control, but they were not significant (Figure 6.18) (Table 6.20). The Wash assay viability from wells with 5mg/ml silver modified GIC was significantly lower than from non-modified GIC but not from control wells. While viability from wells with 10mg/ml were significantly higher than control wells and 5mg/ml silver modified GIC (Figure 6.18) (Table 6.21). The viability of bacterial cells on 5mg/ml silver modified GIC was less than non-modified GIC but was not significant, but bacterial cells viability over 10mg/ml silver modified GIC was significantly higher than both non-modified GIC and 5mg/ml silver modified GIC (Figure 6.18) (Table 6.22).

In comparison to CFU assay, CFUs gave more detailed analysis on the effect of additives on to the colonisation of each species of the model. Logically MTT assay results have to be compared to Mean Total CFUs from each output as it is a measure of the total metabolic activity of each parameter. Aerobically, MTT and CFUs floating assay from wells around either silver modified or non-modified GIC were less than control, but MTT activity around GIC disks was higher than around silver modified GIC whereas CFUs around 5mg/ml silver modified GIC was higher than others. Statistically CFUs from floating assay was not significantly different, but with MTT activity around both silver

modified GIC was significantly lower than control (Figure 6.7and 6.17). Results from wash output using both assays were almost comparable, in both assays wash output from either silver modified GIC was significantly lower than control (Figure 6.8 and 6.17). Regarding the most important output (Intimately attached) from both assays it was confirmed that colonisation and metabolic activity of biofilms attached to Silver modified GIC disks after 24Hrs was significantly lower than non-modified GIC (Figure 6.9 and 6.17).

While under anaerobic conditions, from floating outputs, both assays showed a significantly higher bacterial colonisation and activity around 10mg/ml silver modified GIC than control. But CFUs were lowest around non-modified GIC whereas metabolic activity in control wells was lowest. This increase in either metabolic activity or CFUs around silver modified GIC may be related to the apparent community shift and domination of commensal *N. subflava* which has less virulence effect than *S. mutans* and *S. oralis* (Figure 6.12 and 6.18). Also assays from wash output showed a significant increase in both metabolic activity and colonisation from wells with 10mg/ml silver modified GIC compared to all other wells. Metabolic activity from wells with 5mg/ml silver modified wells was significantly lower than wells with non-modified GIC but this was not significant with CFUs assay (Figure 6.13 and 6.18). Lastly both assays confirmed the significant increase in colonisation and metabolic activity of biofilms attached to 10mg/ml silver modified GIC, and decrease in colonisation and metabolic activity of biofilms attached to 5mg/ml silver modified GIC, but it was not statically significant (Figure 6.14 and 6.18).

In conclusion, MTT assay helped in assessing the antimicrobial activity of bacterial community formed over GIC disks. Together MTT with CFUs confirmed the antibacterial activity of silver enhanced GIC after 24Hrs aerobic and anaerobic incubation. However,

CFUs because of the use of selective agar plates, additional analysis resulted for each species involved in the community.

6.5 DNA Quantity and Quality of Early Coloniser Biofilm Formed on Silver Modified and Non-Modified GIC after 24h Aerobic and Anaerobic Incubation Using Boiling and DNeasy Kit

While we were addressing the PCR experiments, attempts had been made to isolate the intimately attached biofilm DNA after 24Hrs incubation either aerobically or anaerobically. Despite multiple trials, failure to collect pellets out of the intimately attached biofilm occurred because of small bacterial numbers which remained attached to disk surfaces after the washing series, even if the disks were washed only one time only.

That resulted in deciding to isolate the whole biofilm and try to compare it between samples

6.5.1 Materials and Methods

6.5.1.1 Bacterial Species; *S. mutans , S. oralis* and *N. subflava.*

6.5.1.2 Growth Medium; CB and DMM.

6.5.1.3 Solutions

- Additives: silver solutions; 5mg/ml and 10mg/ml.
- PBS (Phosphate buffered saline) (SIGMA # P-4417, Sigma-Aldrich Company Ltd. Dorset, England).
- DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany).
- Sterile distilled water.

6.5.1.4 Specimens

36 disks in total were prepared as in section 4.1.3.1, 12 non-modified GIC disks, and 24 silver modified GIC disks (12 for each silver concentration to be evaluated).

6.5.1.5 Culturing methods and growth conditions

The same culturing and growth conditions as in section 5.9.1.3 were followed, except in the arrangement of modified and non-modified disks through the wells; for each experiment 36 disks were tested, 12 for each silver solution and 12 non-modified GIC. Half of each state specimens were incubated aerobically at 37ºC and the other half were under anaerobic conditions and both for 24Hrs.

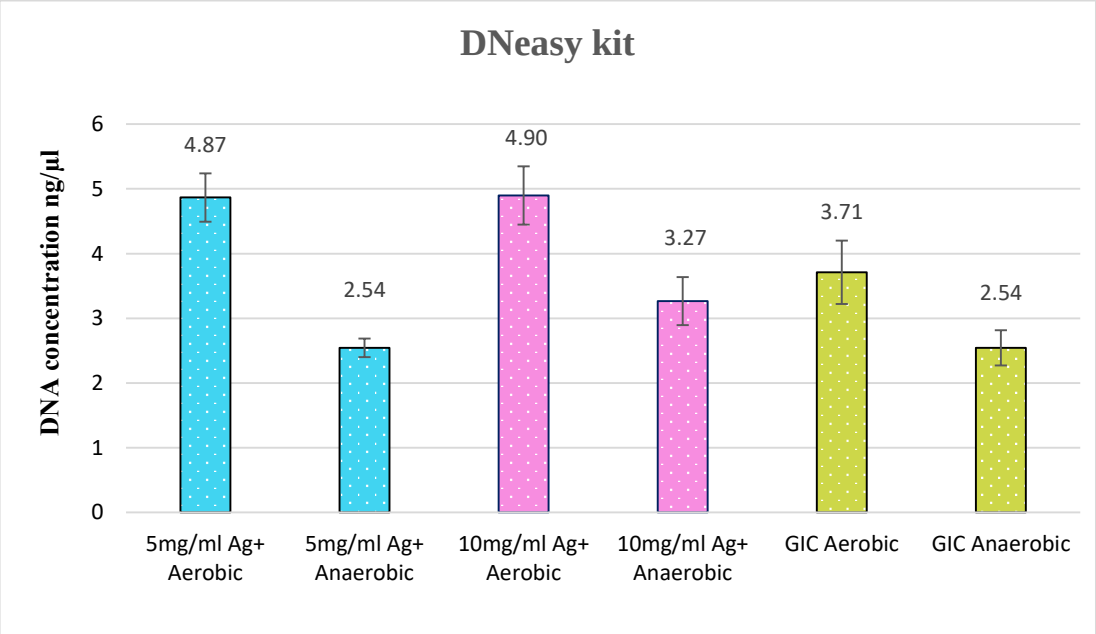
After 24Hrs, disks after aerobic incubation carrying 3D biofilm were carried gently, 3 specimens from both silver modified and non-modified were inserted in labelled Eppendorf tubes containing 1 ml of sterile distilled water. Then they were sonicated and vortexed in sonication bath at 40 KHz for 5 minutes to detach the biofilm from disks. After that DNA was isolated using the Boiling method using the protocol described in section 5.10.1.1. Similar methods were used with discs resulted from anaerobic incubation.

DNA from the rest of the specimens was isolated using DNeasy Kit using manufacturer's protocol after biofilms were collected by sonication and vortexing in sonication bath at 40 KHz for 5 minutes.

- This experiment was repeated three times and DNA was assayed for quantity and quality using spectrophotometer (Nanovue, General Electrics healthcare, Sweden).

6.5.2 Results

Graphs in (Figure 6.19) represents the DNA quantity (Mean±SEM) of the whole biofilm from silver modified and non-modified GIC after 24Hrs aerobic and anaerobic incubation. Comparing the efficiency of Boiling and DNeasy Kit DNA isolation methods.



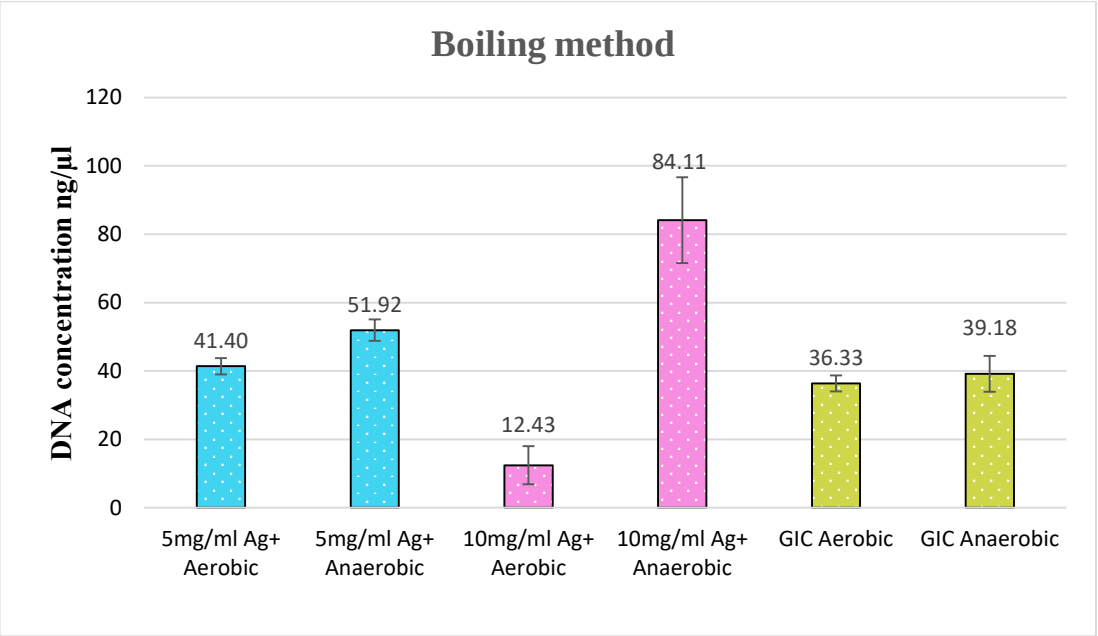


Figure 6.19 DNA concentration (Mean ± SEM *n* =9) in ng/µl of the whole biofilm from silver modified and non-modified GIC surfaces of after 24Hrs incubation aerobically and anaerobically, DNA samples were isolated using DNeasy kit and Boiling methods.

6.5.3 Discussion

Generally, DNA quantities isolated using the Boiling method were significantly higher than quantities isolated using DNeasy kit (Figure 6.19). This may be related to the samples resulted from Boiling method being overestimated with the presence of RNA as mentioned in section (5.10.1.3). In addition to the possibility of incomplete bacterial lysis with DNeasy isolation because as mentioned before a specific number of cells is needed to efficiently isolate the whole DNA with DNeasy kit.

As a result of slower growth under anaerobic conditions compared to aerobic which was confirmed with results from CFUs and MTT assay, DNA quantities must be less from anaerobic conditions, this finding was reflected from DNA samples resulted from DNeasy kit rather than boiling which showed inconsistent results (Figure 6.19). In addition, DNA quantities from 10mg/ml silver modified disks after anaerobic incubation were significantly higher than from aerobic conditions from samples isolated using Boiling method and that finding was opposite to results from CFUs and MTT.

No statistical difference was noticed between the quantities of DNA isolated from silver modified disks and non-modified ones either aerobically or anaerobically using DNeasy kit (Appendix 2 section 10.2.4 for details), which may be related to the whole biofilm being taken without washing and the results were compromised by the presence of loosely attached bacteria. Consequently, the washing series is important to overcome batch culture limitation in the lack of saliva shear force.

In regards to the Boiling method, DNA quantities were significantly different between samples taken from 10mg/ml silver modified compared to 5mg/ml silver modified and non-modified GIC either aerobically or anaerobically (Appendix 2 section 10.2.4). Thses results also may be related to the same reason of taking the whole biofilm rather than what was intimately attached.

In conclusion, despite the smaller DNA quantities resulting from DNeasy kit isolation, results were more realistic than the Boiling method. But because it was needed to take the whole unwashed biofilm to have sufficient bacterial cells for DNA isolation, results couldn't be compared to results either from CFUs nor MTT. Consequently, PCR and qPCR, may not have additional value to this project specifically within 24Hrs, but the development of specific primers and relative quantification of the three species could be useful to another fields e.g. pathogenic bacterial organism identification from oral saliva (Bartlett and Stirling, 2003). of interest or if the biofilm is going further toward maturation after 72Hrs incubation where there should be more bacterial cells attached to restorative materials surfaces.

6.6 The Longevity of Antibacterial Activity of Silver Additives after 48hours under Anaerobic Conditions

As a result of early colonisers microbial community shift toward the colonisation and domination of *N. subflava* after 24Hrs anaerobic incubation in experiment 6.3, further investigations were needed to identify if that finding possibly happened because streptococci were growing relatively slower than *N. subflava*. In addition, we wanted to investigate the regeneration of early coloniser species and the longevity of the silver additives antimicrobial activity on the growth of early coloniser model species after 48Hrs anaerobic incubation. To address that the following experiment was conducted.

6.6.1 Materials and Methods

Same as in experiment (6.3), but the incubation time was 48Hrs anaerobically.

6.6.2 Results

CFUs assay was used to analyse the results and compare it to results from experiment 6.3, The planktonic growth around disks (Floating), what was loosely attached to the well surfaces (Wash) and what remained attached to disk surfaces after 5 times serial washes (Intimately attached) are demonstrated in Figures 6.20-6.22.

SEM images for the Intimately attached biofilm are presented in (Figure 6.23).

The same statistical analysis was conducted for the data set using Friedman's two-way ANOVA by rank test were $p < 0.05$ were considered significant.

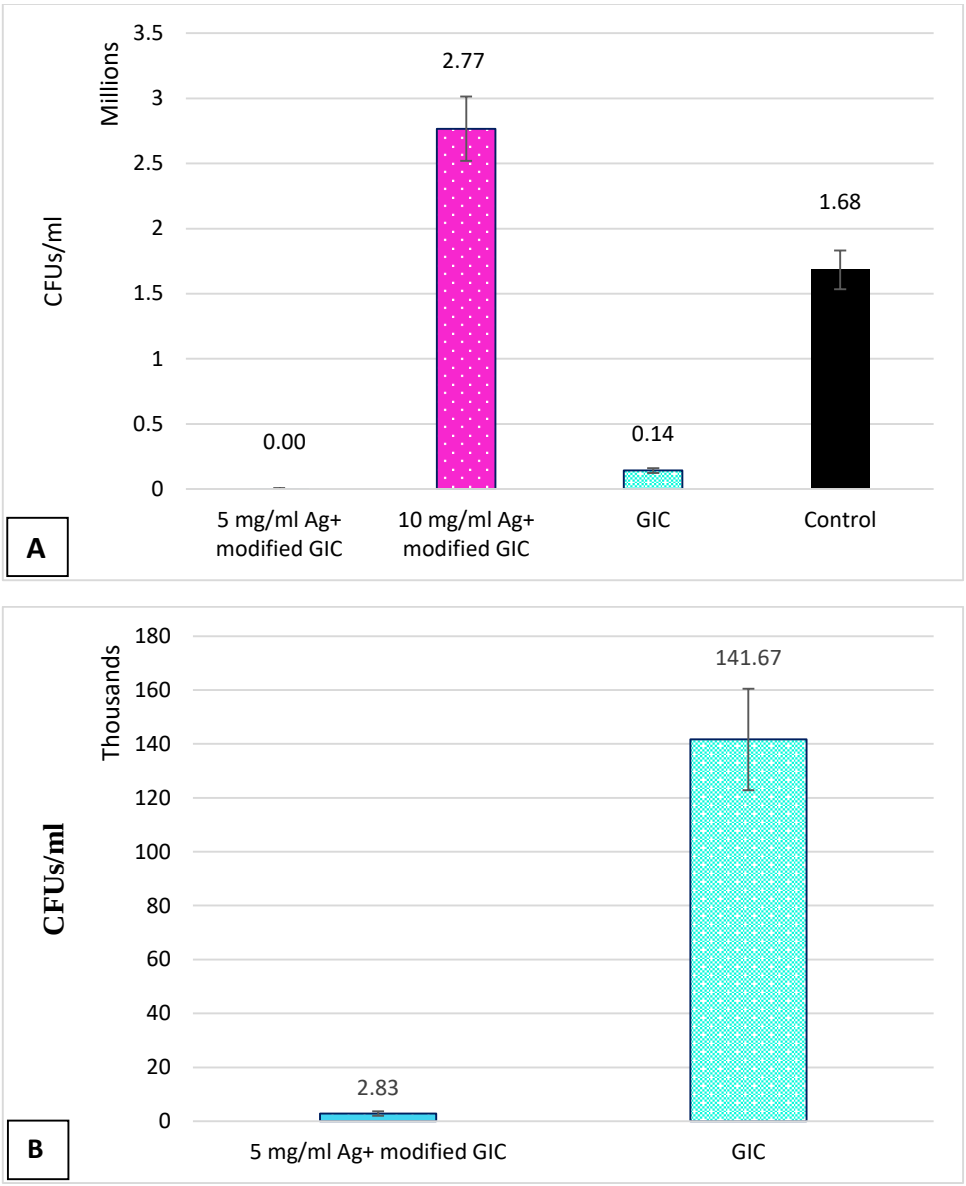


Figure 6.20 Graphs represent viable bacterial count (CFUs) (Mean ± SEM *n* =6) for the planktonic bacterial growth around disks either silver modified or non-modified (Floating) in Total after anaerobic incubation for 48 Hrs. Graph B is a highlighted part of graph A, *S. oralis* was the only species detected, *S. oralis* CFUs was significantly higher in wells with 10mg/ml silver modified GIC compared to wells with 5 mg/ml silver modified GIC, and also was significantly lower in wells with 5mg/ml silver modified GIC compared to control and non-modified GIC (Friedman's two-way ANOVA by rank test) ($p < 0.05$).

Table 6.23 Pairwise comparison of the Total Floating *S. oralis* CFUs showing a highly significant difference between wells contains 10mg/ml silver modified GIC in comparison to 5mg/ml silver modified GIC, 5mg/ml silver modified GIC in comparison to control (* *p <0.05).*

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	***0.001****
10mg/ml silver modified GIC – ChemFil Superior GIC	0.083
10mg/ml silver modified GIC – Control	1.000
5mg/ml silver modified GIC – ChemFil Superior GIC	***0.014****
5mg/ml silver modified GIC – Control	***0.022****
ChemFil Superior GIC - Control	0.705

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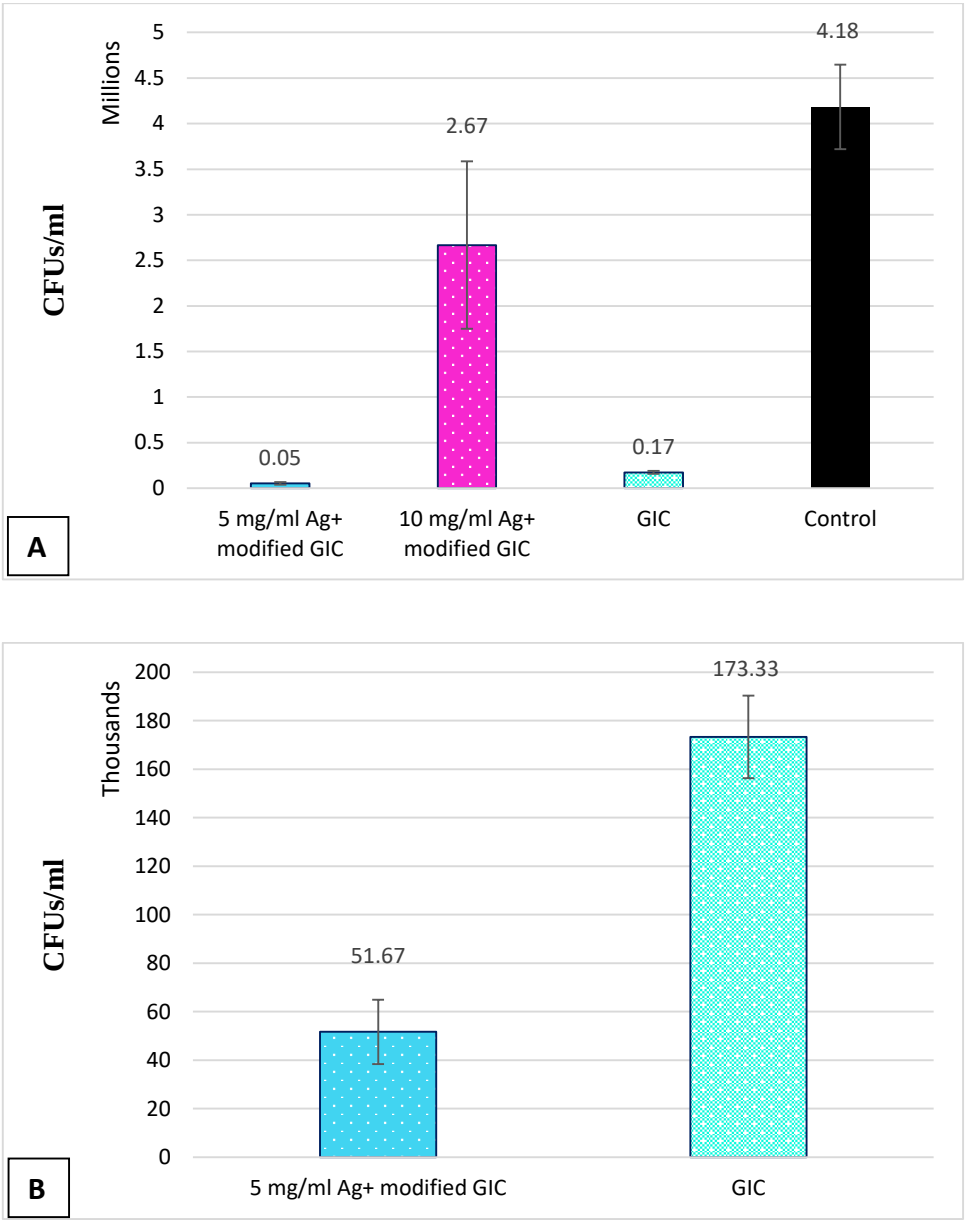


Figure 6.21 Graphs represent viable bacterial count (CFUs) (Mean ± SEM *n* =6) assayed from well surfaces washing (Wash) after anaerobic incubation for 48 Hrs; Graph B is a highlighted part of graph A, *S. oralis* was the only species detected, *S. oralis* CFUs was significantly higher in wells with 10mg/ml silver modified GIC compared to wells with 5 mg/ml silver modified GIC, and also was significantly lower in wells with 5mg/ml silver modified GIC compared to control and non-modified GIC (Friedman's two-way ANOVA by rank test) ($p < 0.05$).

Table 6.24 Pairwise comparison of the Total Wash *S. oralis* CFUs showing a highly significant difference between wells contains 10mg/ml silver modified GIC in comparison to 5mg/ml silver modified GIC, 5mg/ml silver modified GIC in comparison to control (* *p <0.05).*

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	***0.022****
10mg/ml silver modified GIC – ChemFil Superior GIC	0.705
10mg/ml silver modified GIC – Control	1.000
5mg/ml silver modified GIC – ChemFil Superior GIC	***0.014****
5mg/ml silver modified GIC – Control	***0.001****
ChemFil Superior GIC - Control	0.083

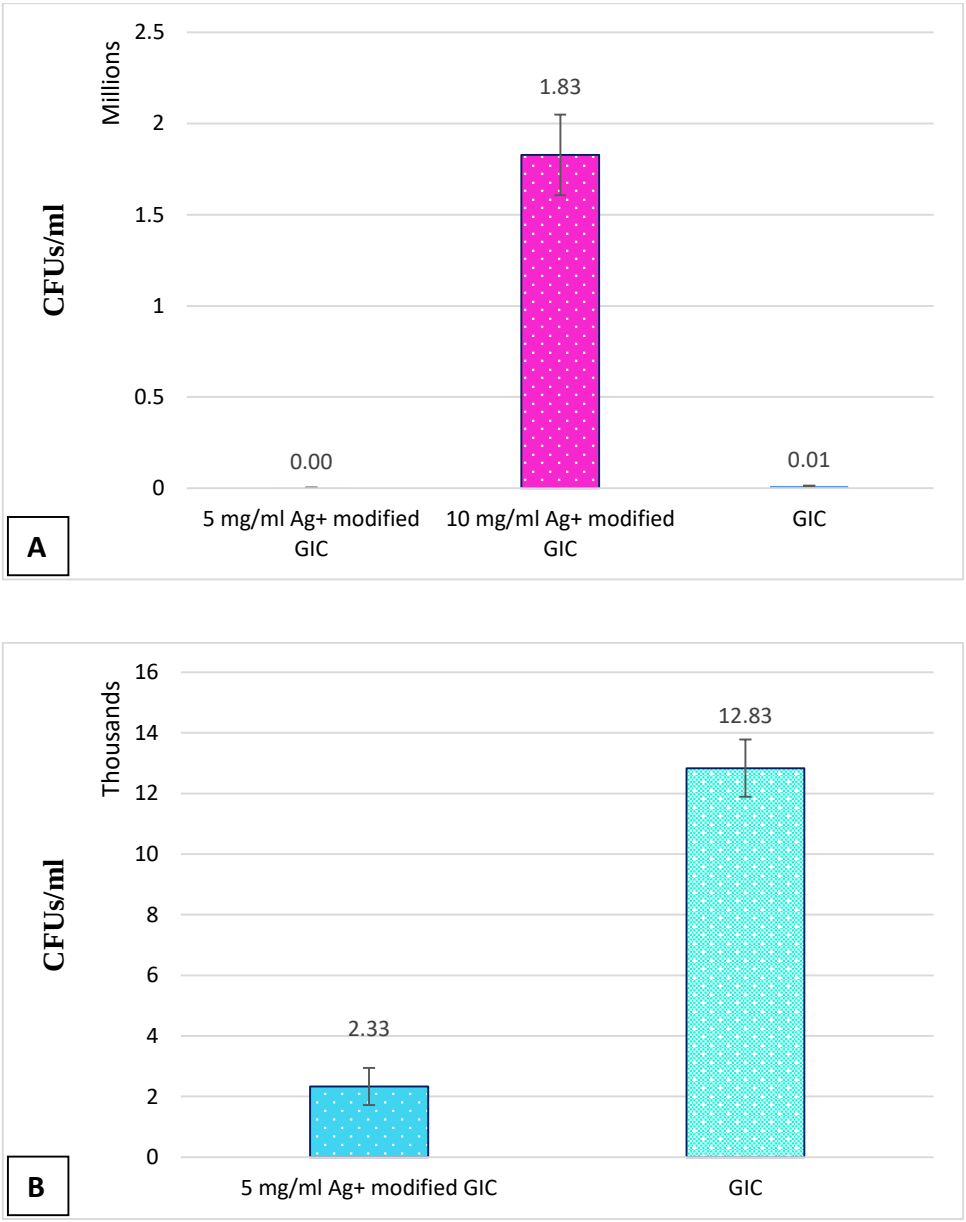


Figure 6.22 Graphs represent viable bacterial count (CFUs) (Mean ± SEM *n* =6) assayed from disk surfaces after anaerobic incubation for 48 Hrs. Graph B is a highlighted part of graph A, *S. oralis* was the only species detected. *S. oralis* CFUs was significantly higher from 10mg/ml silver modified GIC disks compared to 5mg/ml silver modified GIC disks, and it was significantly lower from 5mg/ml silver modified GIC disks compared to non-modified GIC (Friedman's two-way ANOVA by rank test) ($p < 0.05$).

Table 6.25 Pairwise comparison of the Intimately Attached CFUs showing a highly significant difference bacterial cells counts Intimately attached to 10mg/ml silver modified GIC in comparison to 5mg/ml silver modified GIC, and between 5mg/ml silver modified GIC in comparison non-modified GIC (* *p <0.05).*

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	***0.002****
10mg/ml silver modified GIC – ChemFil Superior GIC	0.250
5mg/ml silver modified GIC – ChemFil Superior GIC	***0.014****

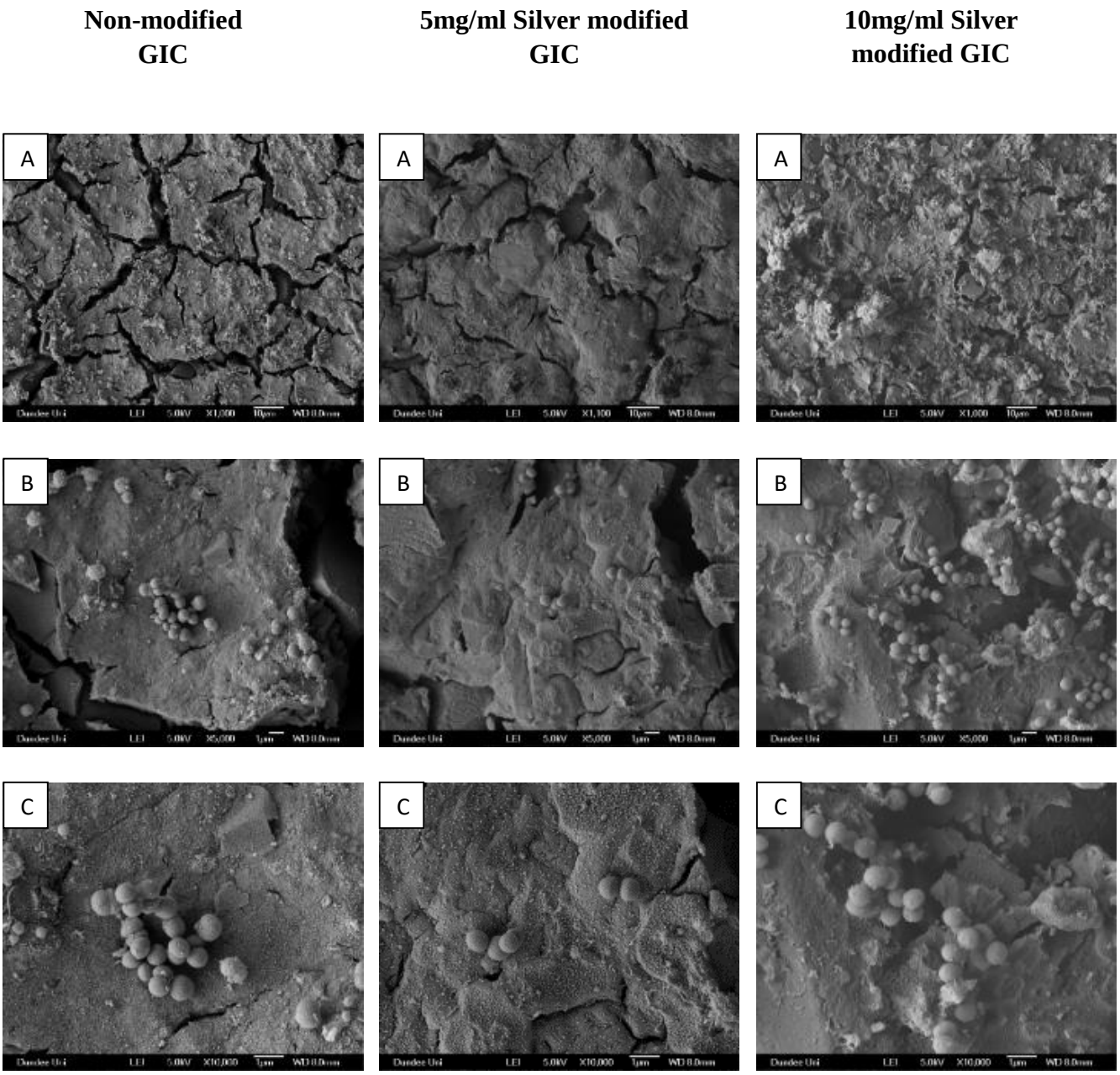


Figure 6.23 SEM images for the Intimately attached biofilm to silver modified and non-modified GIC after 48Hrs incubation under anaerobic conditions at three magnifications; (A) 1000X, (B) 5000X and (C) 10000X. 5mg/ml silver modified GIC reduced the colonisation while 10mg/ml silver modified GIC enhanced it compared to non-modified GIC.

6.6.3 Discussion

DMM could support the growth of early coloniser model species up to 48 Hrs as tested before in experiment (5.2). Consequently, early coloniser species supposed to continuously grow despite the possible nutrients depletion in wells closed system.

After having a shift in *in vitro* biofilm community from *S. oralis* being the dominant coloniser aerobically to *N. subflava* being dominant anaerobically, this experiment was designed to question if the growth of *N. subflava* was dominating and masking the slow growth rate of *S. oralis* and *S. mutans.*

The modification was to increase the incubation time to 48Hrs to assess the growth and regeneration of the model species, also the idea of changing the media after 24Hrs with fresh DMM was not feasible because the resultant Floating and Washed community would be different and couldn't be compared with the previous results.

Interestingly *S. oralis* was the only species retrieved from all outputs, assuming *N. subflava* and *S. mutans* had either died or they were below the detection limit of CFUs assay.

The effect of silver additives to GIC was also clear, from Floating output in (Figure 6.20) 10mg/ml silver modification of GIC again increased the colonisation of *S. oralis* significantly, while 5mg/ml silver addition decreased it significantly compared to non-modified GIC (Table 6.23). The growth around 10mg/ml silver modified GIC disks was more than the control wells which indicated either something in the modified formulation that may enhanced the growth of model species, or PVA retarded the antibacterial activity of silver. To explain this another experiment was conducted in section (6.7).

In regards to the loosely attached assay (Wash), *S. oralis* count from wells with 10mg/ml silver modified GIC disks was significantly higher than from wells with non-modified

GIC and 5mg/ml silver modified GIC disks. Also the count from wells with 5mg/ml silver modified GIC disks was significantly lower than wells with non-modified GIC (Figure 6.21) (Table 6.24), indicating that the presence of additive increased the colonisation with 10mg/ml silver modification and decreased it significantly with 5mg/ml silver modification.

10mg/ml silver addition to GIC increased the colonisation of *S. oralis* on disk surfaces significantly, on the other hand 5mg/ml silver addition decreased the colonisation significantly compared to non-modified GIC disks (Figure 6.22) (Table 6.25).

SEM for the Intimately attached biofilm confirmed the finding of reducing colonisation on 5 mg/ml silver modification to GIC compared to non-modified GIC, however 10mg/ml silver additive enhanced the colonisation after 48Hrs of anaerobic incubation (Figure 6.23).

In conclusion, 5mg/ml silver addition to GIC significantly enhanced the antibacterial activity of GIC either aerobically or anaerobically within 48Hrs by decreasing the colonisation of early coloniser species.

6.7 The Effect of PVA Addition to Silver on the Attachment and Survival of Early Coloniser Model after 24 Hrs under Anaerobic Conditions

10mg/ml silver modified GIC enhanced the growth of early coloniser model species after 24Hrs incubation and enhanced *S. oralis* growth after 48Hrs under anaerobic conditions. To assess if that happened because PVA in 10mg/ml silver modified formulation enhanced the growth of model species, or PVA retarded the antibacterial activity of silver, this experiment was conducted to identify the antibacterial effect of PVA addition to GIC.

6.7.1 Materials and Methods

Same as in experiment (6.3), but PVA at 5mg/ml was added to GIC powder to make GIC disks and tested against 10mg/ml silver modified GIC and non-modified GIC under anaerobic conditions for 24Hrs.

6.7.2 Results

CFUs assay was used to analyse the results and compare it to results from experiment 6.3, The planktonic growth around disks (Floating), what was loosely attached to well surfaces (Wash) and what remained attached to disk surfaces after 5 times serial washes (Intimately attached) are demonstrated in Figures 6.24-6.26.

The same statistical analysis conducted for the data set using Friedman's two-way ANOVA by rank test and $p < 0.05$ was considered to be significant.

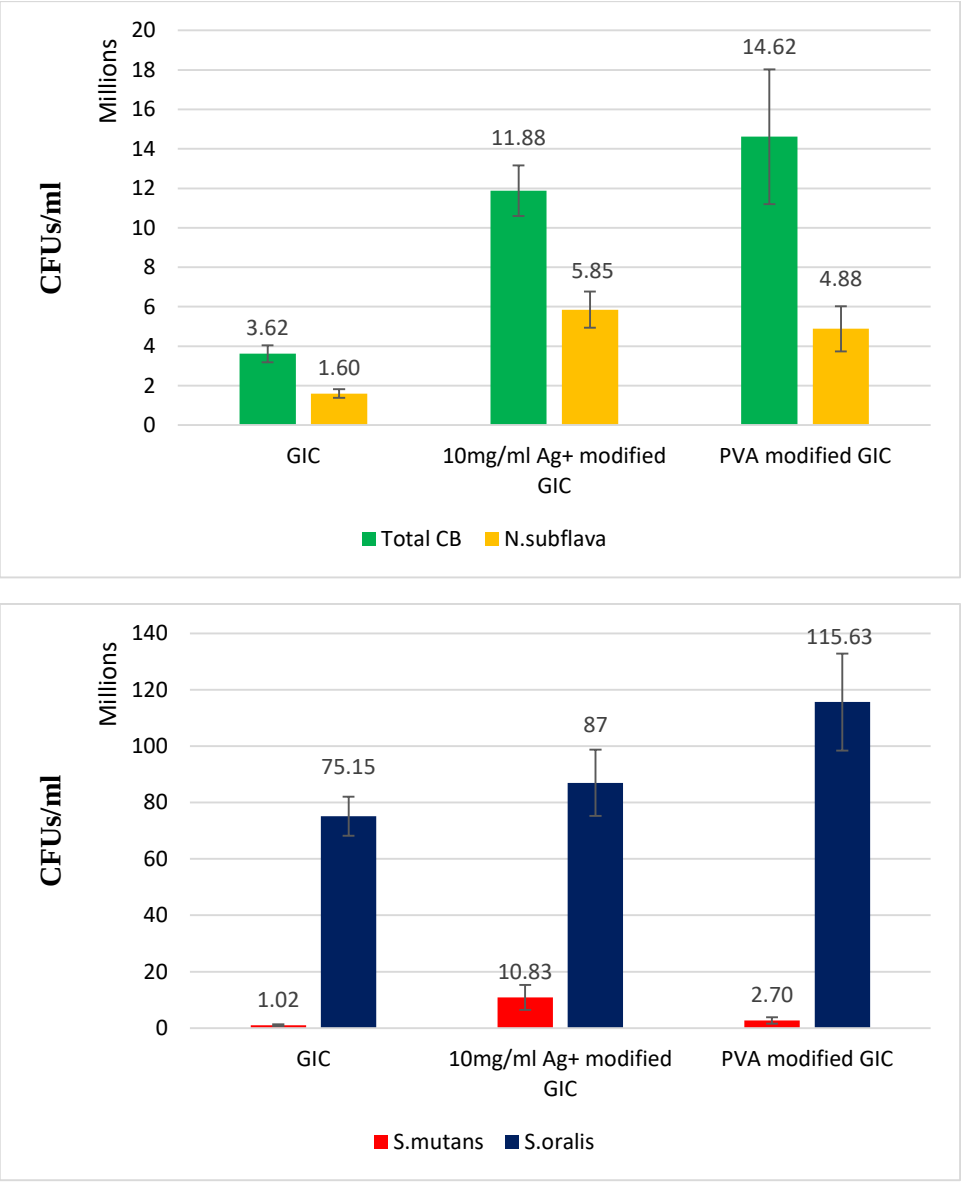


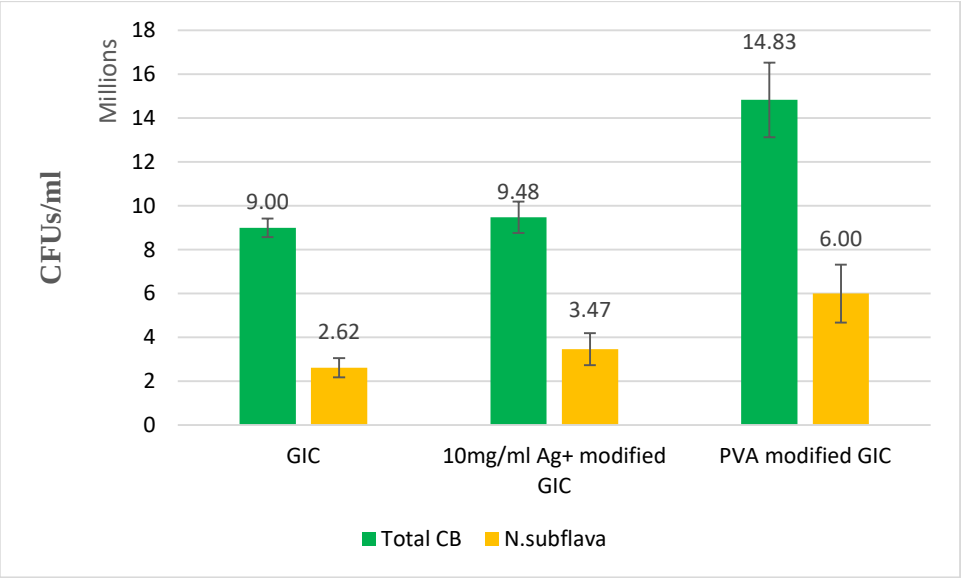
Figure 6.24 Graphs represent viable bacterial count (CFUs) (Mean ± SEM *n* =6) in millions for the planktonic bacterial growth around disks (Floating) in Total and for each of the three species after anaerobic incubation for 24 Hrs. *N. subflava* counts was 42% on average of Total, Total CFUs and *N. subflava* CFUs was significantly higher in wells with PVA modified GIC disks compared to non-modified GIC (Friedman's two-way ANOVA by rank test) ($p < 0.05$).

Table 6.26 Pairwise comparison of the Total Floating CFUs showing a highly significant difference between wells with PVA modified GIC in comparison non-modified GIC (* *p <0.05).*

Sample1-Sample2	Significance
10mg/ml silver modified GIC – PVA modified GIC	1.000
10mg/ml silver modified GIC – ChemFil Superior GIC	0.063
PVA modified GIC – ChemFil Superior GIC	***0.012****

Table 6.27 Pairwise comparison of the *N. subflava* Floating CFUs showing a highly significant difference between wells with 10mg/ml silver modified GIC in comparison non-modified GIC (* *p <0.05).*

Sample1-Sample2	Significance
10mg/ml silver modified GIC – PVA modified GIC	0.745
10mg/ml silver modified GIC – ChemFil Superior GIC	***0.018****
PVA modified GIC – ChemFil Superior GIC	0.337



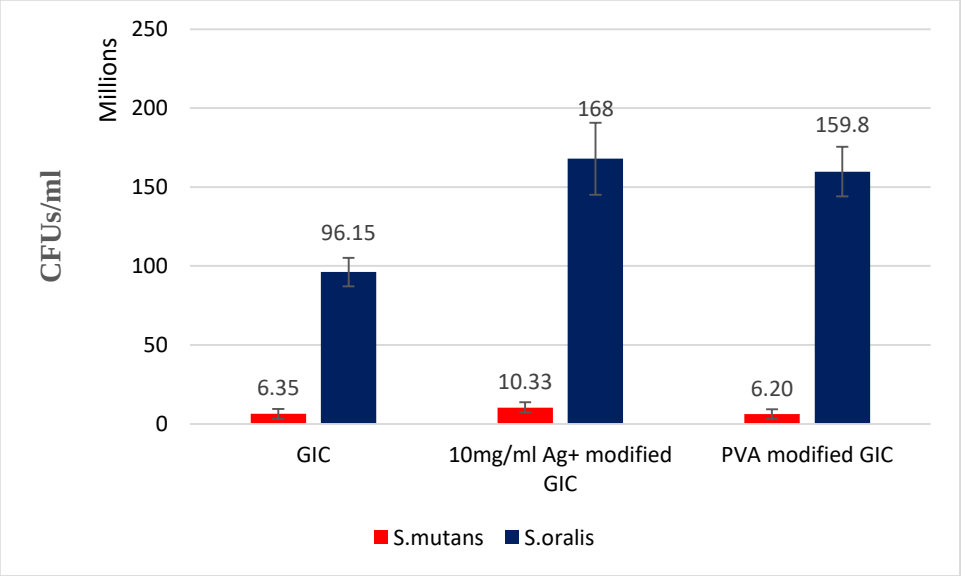
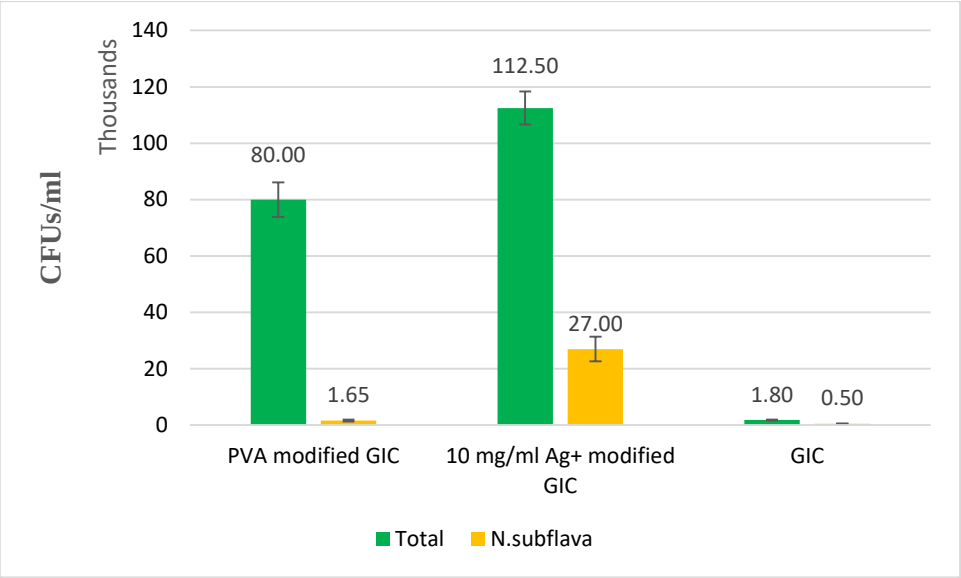


Figure 6.25 Graphs represent viable bacterial count (CFUs) (Mean ± SEM *n* =6) in millions in Total and for the three species assayed from well surfaces washing (Wash) after anaerobic incubation for 24 Hrs. *N. subflava* counts was 35.2% on average of Total. *S. oralis* Wash CFUs significantly higher in wells contains 10mg/ml silver modified GIC and PVA modified GIC compared to non-modified GIC.

Table 6.28 Pairwise comparison of the *S. oralis* Wash CFUs showing a highly significant difference between wells with PVA modified GIC, and 10mg/ml silver modified GIC in comparison non-modified GIC (* *p <0.05).*

Sample1-Sample2	Significance
10mg/ml silver modified GIC – PVA modified GIC	1.000
10mg/ml silver modified GIC – ChemFil Superior GIC	***0.018****
PVA modified GIC – ChemFil Superior GIC	***0.042****



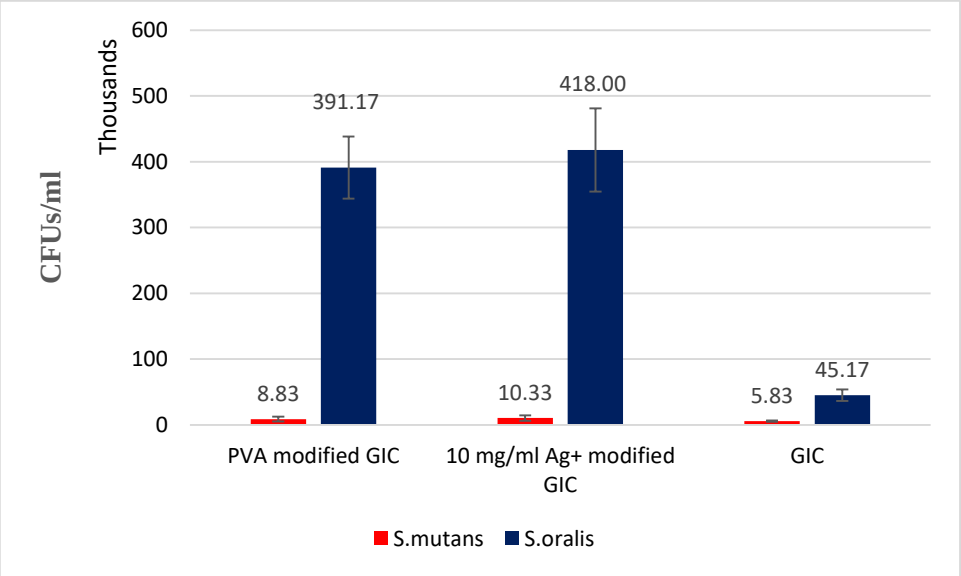


Figure 6.26 Graphs represent viable bacterial count (CFUs) (Mean ± SEM *n* =6) in thousands in Total and for the three species assayed from disk surfaces (Intimately Attached) after anaerobic incubation for 24 Hrs. Total CFUs and *N. subflava* CFUs was significantly higher from disks with 10mg/ml silver modified GIC compared to non-modified GIC. *S. oralis* CFUs was significantly higher from disks with either PVA or 10mg/ml modification in comparison to non-modified GIC (Friedman's two-way ANOVA by rank test) ($p < 0.05$).

Table 6.29 Pairwise comparison of the Total Intimately attached CFUs showing a highly significant difference between 10mg/ml silver modified GIC in comparison non-modified GIC (*$p < 0.05$).

Sample1-Sample2	Significance
10mg/ml silver modified GIC – PVA modified GIC	0.745
10mg/ml silver modified GIC – ChemFil Superior GIC	***0.004****
PVA modified GIC – ChemFil Superior GIC	0.130

Table 6.30 Pairwise comparison of the *N. subflava* Intimately attached CFUs showing a highly significant difference between 10mg/ml silver modified GIC in comparison non-modified GIC Disks (* $p < 0.05$).

Sample1-Sample2	Significance
10mg/ml silver modified GIC – PVA modified GIC	0.250
10mg/ml silver modified GIC – ChemFil Superior GIC	***0.002****
PVA modified GIC – ChemFil Superior GIC	0.250

Table 6.31 Pairwise comparison of the *S. oralis* Intimately attached CFUs showing a highly significant difference between PVA modified GIC, and 10mg/ml silver modified GIC in comparison non-modified GIC (*$p < 0.05$).

Sample1-Sample2	Significance
10mg/ml silver modified GIC – PVA modified GIC	1.000
10mg/ml silver modified GIC – ChemFil Superior GIC	***0.028****
PVA modified GIC – ChemFil Superior GIC	***0.028****

6.7.3 Discussion

Polyvinyl alcohol polymer (PVA) has been widely described in the literature as a stabiliser for Silver nanoparticles AgNPs. It also plays an important role in shape-controlled seeded-growth and colloidal stability. However, theoretical aspects of the stabilising mechanism of PVA are still poorly understood (Mahmoud, 2015).

Addition of PVA as a stabiliser for silver citrate/citric acid complexes is not reported in the searched literature, but it was chosen because of failures to stabilise silver citrate complexes in higher concentration above 5mg/ml with low acidity. Also because it is a suitable host matrix due to its high mechanical strength, water-solubility so it can be mixed with ChemFil Superior powder, good environmental stability, easy process ability, besides, it is semi crystalline, fully biodegradable, bio compatible, and nontoxic (Kyrychenko et al., 2017).

10mg/ml silver solution contains PVA in its formulation at a concentration of 5mg/ml. It showed a higher stability than 13mg/ml silver solution over the experimental period of time (6 months). Despite its addition to GIC enhanced the antibacterial activity aerobically after 24Hrs, but anaerobically the addition increased the colonisation after 24Hrs and 48Hrs. When 5mg/ml PVA solution was added to GIC and tested with the early coloniser model anaerobically the following finding resulted.

Floating CFUs in total showed a significant increase in wells with PVA modified GIC disks compared to non-modified GIC disks, although CFUs in total increased in wells with 10mg/ml silver modified GIC compared to wells with non-modified GIC, but it was not statistically significant (Figure 6.24) (Table 6.26). *N. subflava* percentage from total counts was near 42% on average compared to 57.52% in experiment 6.3. Also *N. subflava* counts were significantly higher in wells with 10mg/ml silver modified GIC compared to

non-modified GIC disks (Figure 6.24) (Table 6.27). Although there was an increase in S. oralis counts in wells with silver modified GIC, it was not statistically significant, same as for *S. mutans* counts (Figure 6.24).

Wash CFUs also revealed an increase in colonisation of the three species in Total, *S. oralis* and *N. subflava* from wells with PVA and 10mg/ml silver modified GIC disks, but the increase in *S. oralis* was only significant (Figure 6.25) (Table 6.28).

Regarding colonisation on disk surfaces, Total CFUs and *N. subflava* CFUs was significantly higher from disks with 10mg/ml silver modified GIC compared to non-modified GIC (Figure 6.26) (Tables 6.29 and 6.30). PVA and 10mg/ml addition to GIC significantly increased *S. oralis* counts compared to non-modified GIC disks but not *S. mutans* counts (Figure 6.26) (Table 6.31).

From all previous findings within the parameters of our experiment PVA enhanced the colonisation of the model species anaerobically after 24Hrs when it was in 10mg/ml silver formulation or without. This could be explained by oxygen deprived conditions; the coloniser species may utilise PVA as a source of energy, it is proven that PVA with its carbon–carbon backbone, being water soluble can be biodegraded under both aerobic and anaerobic conditions (Suzuki et al. 1973; Watanabe et al. 1976; Sakazawa et al. 1981; Shimao et al. 1983; Mori et al. 1996; Matsumura et al. 1993). Species that degrade PVA include Pseudomonas O-3 (Suzuki et al. 1973), Pseudomonas sp. (Shimao et al. 1984, 1986, 1996; Watanabe et al. 1975, 1976; Sakai et al. 1981, 1985a, b), Alcaligenes faecalis (Matsumura et al. 1993) and Bacillus megaterium (Mori et al. 1996). Among fungi, Penicillium sp. has also been reported to produce PVA-degrading enzyme (Qian et al. 2004).

Although early coloniser species are not part of the proven species which degrade PVA, and PVA incorporation with AgNPs didn't report any enhanced colonisation in previous studies section (2.5.1.1). Part of the future work from this project will be concerned in investigations on the effect of PVA on growth of bacterial species.

In conclusion, PVA addition to GIC enhanced the colonisation of the early coloniser model under anaerobic conditions.

6.8 Determination of the Physical Properties of the Glass Ionomer Cements at Baseline and After Silver Additions

At baseline and following the additions of silver solutions the following physical properties, of potential significance for clinical durability were determined using protocols previously published for testing those properties (Salem et al., 2016).

- Surface hardness (5 specimens).
- Compressive strength (20 specimens).
- Adhesive shear bond strength to human dentine (15 specimens).

6.8.1 Specimens Fabrication and Storage

All baseline specimens were mixed according to the manufacturer's instructions and silver modified specimens were constructed as mentioned in section 4.1.3.2.

Silver modified GIC disks were prepared by mixing ChemFil powder with each silver solution at 1:1 Powder/Liquid ratio i.e. replacement of water by silver solution. However, in 10mg/m silver modified GIC mix the ratio was 1:2 powder/liquid ratio, which was needed to have a uniform mix without powder residues. Once fabricated all specimens were stored in distilled water at 37 °C in a thermostatically controlled oven for one week prior to testing.

6.8.2 Compressive Strength

6.8.2.1 Materials and Methods

- **Specimens**

  40 Silver modified GIC specimens, 20 for each silver solution evaluated.

  20 Non-silver modified GIC specimens.

- **Specimen Testing**

An Instron Universal testing machine (Model 4469, Instron Ltd., High Wycombe,UK) (Figure 6.27) used to perform the test. The testing procedures described in this thesis utilised standard methodology used in the Dental materials laboratories in Dundee Dental School where the tests were undertaken.

Figure 6.27 An Instron Universal testing machine.

The compressive strength was determined at a cross head speed of 1 mm min^{-1}. Prior to testing the length and diameter of each specimen was measured using a micrometre. Then the flat end of each specimen was covered with wet circular filter paper disk (Whatman No.1, Whatman International Ltd., Maidstone, UK) as recommended by Baig (Baig et al., 2015). The results for each material and state were expressed as a mean and standard deviation (SD). This data was subject to analysis of variance with *post hoc* testing using the Tukey comparison of means test.

The formula used to determine compressive strength was: mm^2

$$\textbf{Compressive Strength (MPa)} = \frac{\textbf{Force at Failure in Newtons (N)}}{\textbf{Cross Sectional Area of Specimen } (\textbf{mm}^{\textbf{2}})}$$

6.8.2.2 Results

Tables 6.32 - 6.34 give the dimensions (length, diameter) and force at failure for compressive specimens together with their calculated compressive strength.

(Figure 6.28) gives the mean and the standard deviation of the observed compressive strengths for each group.

Table 6.32 Compressive strength of GIC ChemFil Superior measured at 1mm/ min following one week of storage in distilled water.

Specimen Number	Diameter (mm)	Cross Sectional Area (mm^2)	Failure Force (N)	Compressive Strength (MPa)
1	4.053	12.906	668.5	51.79
2	4.096	13.182	970.5	73.62
3	3.961	12.327	776.2	62.96
4	3.933	12.153	760.6	62.58
5	3.978	12.433	911.1	73.27
6	3.973	12.402	979.5	78.97
7	4.083	13.098	1005	76.72
8	3.953	12.277	689.5	56.15
9	4.171	13.669	690.3	50.50
10	4.045	12.855	781.1	60.75
11	4.101	13.214	797.3	60.33
12	3.926	12.110	742.3	61.29
13	3.958	12.308	1011	82.13
14	4.033	12.779	1045	81.77
15	3.916	12.048	1067	88.55
16	4.197	13.840	960.4	69.39
17	4.088	13.130	997.4	75.95
18	4.022	12.710	962.4	75.71
19	4.077	13.060	963.6	73.78
20	4.186	13.767	790.6	57.42
Mean				68.68
SD				10.87

Table 6.33 Compressive strength of 5mg/ml Silver modified GIC ChemFil Superior measured at 1mm/ min following one week of storage in distilled water.

Specimen Number	Diameter (mm)	Cross Sectional Area (mm^2)	Failure Force (N)	Compressive Strength (MPa)
1	4.037	12.805	1651	128.93
2	4.014	12.659	1962	154.98
3	3.985	12.477	1196	95.85
4	4.068	13.002	1326	101.98
5	3.971	12.389	1530	123.48
6	4.097	13.188	1357	102.89
7	4.041	12.830	1565	121.97
8	4.037	12.805	1601	125.02
9	4.093	13.162	1583	120.26
10	4.114	13.298	1497	112.57
11	4.158	13.584	1626	119.69
12	4.001	12.577	1401	111.38
13	4.054	12.913	1592	123.28
14	3.997	12.552	1525	121.48
15	3.975	12.414	1415	113.97
16	4.013	12.653	1587	125.42
17	4.018	12.684	1859	146.55
18	4.087	13.124	1694	129.07
19	4.075	13.047	1487	113.97
20	4.006	12.609	1497	118.72
Mean				120.75
SD				13.71

Table 6.34 Compressive strength of 10 mg/ml Silver modified GIC ChemFil Superior measured at 1mm/ min following one week of storage in distilled water.

Specimen Number	Diameter (mm)	Cross Sectional Area (mm^2)	Failure Force (N)	Compressive Strength (MPa)
1	3.949	12.252	1315	107.32
2	4.092	13.156	1369	104.05
3	4.356	14.908	955.7	64.10
4	4.327	14.710	928.5	63.11
5	4.039	12.817	1267	98.84
6	4.035	12.792	1499	117.17
7	4.003	12.590	1260	100.07
8	4.01	12.634	1136	89.91
9	3.977	12.427	1297	104.36
10	3.95	12.259	1491	121.62
11	4.011	12.640	1416	112.01
12	4.021	12.703	1207	95.01
13	3.996	12.546	1214	96.76
14	3.991	12.514	1384	110.58
15	4.089	13.137	1170	89.06
16	4.273	14.345	949	66.15
17	4.198	13.846	979.9	70.76
18	4.06	12.951	918.1	70.88
19	4.067	12.996	1285	98.87
Mean				93.72
SD				18.43

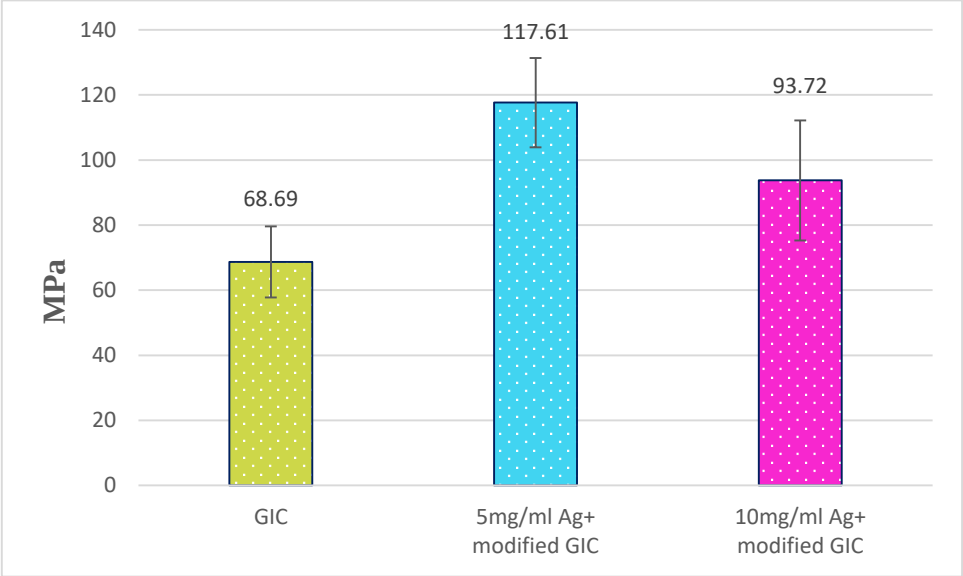


Figure 6.28 Compressive Strength of specimens in Mega Pascal (MPa) for ChemFil Superior, silver modified ChemFil Superior (Mean±SD *n* =20).

The data conformed to a normal statistical distribution as assessed by D'Agostino & Pearson omnibus normality test. Accordingly, an analysis of variance using Tukey comparison of means test was conducted and revealed highly statistically significant difference ($P < 0.0001$) between the values summarised in (Table 6.35) which shows a significant enhanced compressive strength with both silver enhanced formulations.

Table 6.35 Tukey comparison of means of Compressive strength values. Showing a highly significant difference between silver modified GIC and non-modified GIC ($p < 0.0001$).

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	< 0.0001
10mg/ml silver modified GIC – ChemFil Superior GIC	< 0.0001
5mg/ml silver modified GIC – ChemFil Superior GIC	< 0.0001

6.8.3 Adhesive Shear Bond Strength to Dentine

6.8.3.1 Materials and Methods

- **Specimens**

  30 Silver modified GIC specimens, 15 for each concentration of silver solution evaluated.

  15 Non-modified GIC specimens.

- **Specimen Testing**

  A specially constructed jig (Figure 6.29) mounted upon the load cell of an Instron Universal Testing Machine was used to determine the shear bond strengths of the Glass Ionomer Cements to Dentine of sectioned molar teeth for both manufactured and modified form. Specimens were tested to failure. The blade of the assembly was applied, as close as possible, to the cement/tooth interface at a crosshead speed of 0.5 mm min^{-1}.

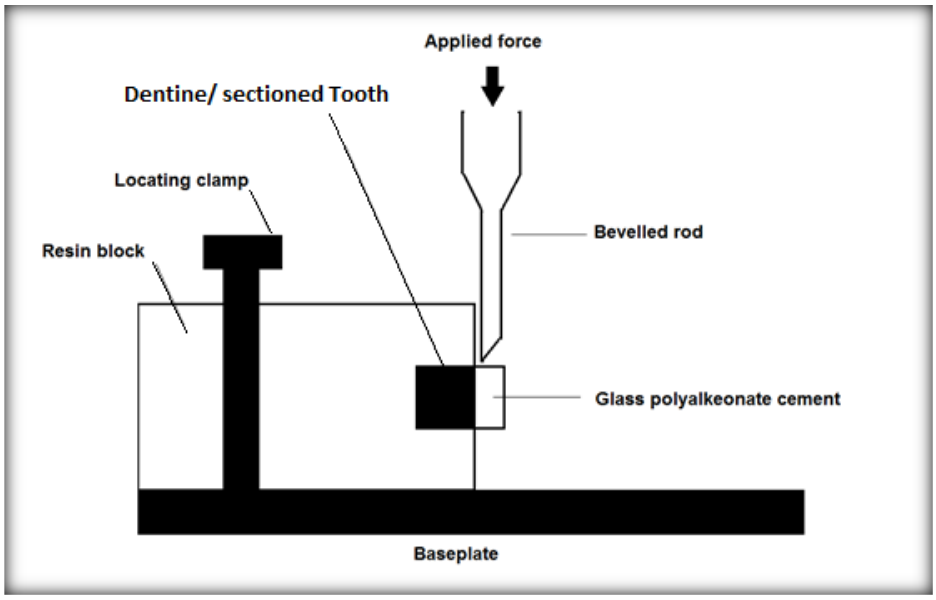


Figure 6.29 Schematic diagram of the adhesive shear strength testing.

(Courtesy of Professor R.G. Chadwick)

The shear bond strength was calculated using the formula:

$$\textbf{Shear Bond Strength (MPa)} = \frac{\textbf{Force at Failure in Newtons (N)}}{\textbf{Bonded Area (mm}^{\textbf{2}}\textbf{)}}$$

Following the confirmation that data followed a normal distribution, it was subject to analysis of variance with *post hoc* testing using the Tukey comparison of means test.

6.8.3.2 Results

Table 6.36 gives the force at failure for Adhesive shear bond strength specimens together with the associated calculated adhesive shear bond strengths.

Figure 6.30 gives the mean and the standard deviation of the observed shear bond strengths for each group.

Table 6.36 Adhesive shear bond strength of silver modified and non-modified ChemFil Superior following one week of storage in distilled water.

	GIC		5 mg/ml		10mg/ml	
Specimen	**Failure Force (N)**	**Shear Bond Strength (MPa)**	**Failure Force (N)**	**Shear Bond Strength (MPa)**	**Failure Force (N)**	**Shear Bond Strength (MPa)**
1	69.8	5.55	89.9	7.15	14.8	1.17
2	43.0	3.42	99.3	7.89	20.1	1.59
3	67.1	5.33	96.2	7.65	14.8	1.17
4	51.0	4.05	124.8	9.92	18.8	1.49
5	45.6	3.62	82.2	6.53	12.1	0.96
6	64.4	5.12	98	7.79	18.8	1.49
7	77.9	6.19	83.2	6.61	26.0	2.06
8	55.0	4.37	106	8.43	14.7	1.16
9	47.0	3.73	76.5	6.08	22.4	1.78
10	38.9	3.09	80.4	6.39	16.0	1.27
11	65.8	5.23	80.5	6.40	19.6	1.55
12	59.1	4.70	71.2	5.66	20.3	1.61
13	63.1	5.01	83.2	6.61	26.1	2.07
14	67.1	5.33	89.9	7.15	16.6	1.32
15	64.4	5.12	95.4	7.58	12.7	1.01
Mean		4.66		7.19		1.45
SD		0.89		1.07		0.34

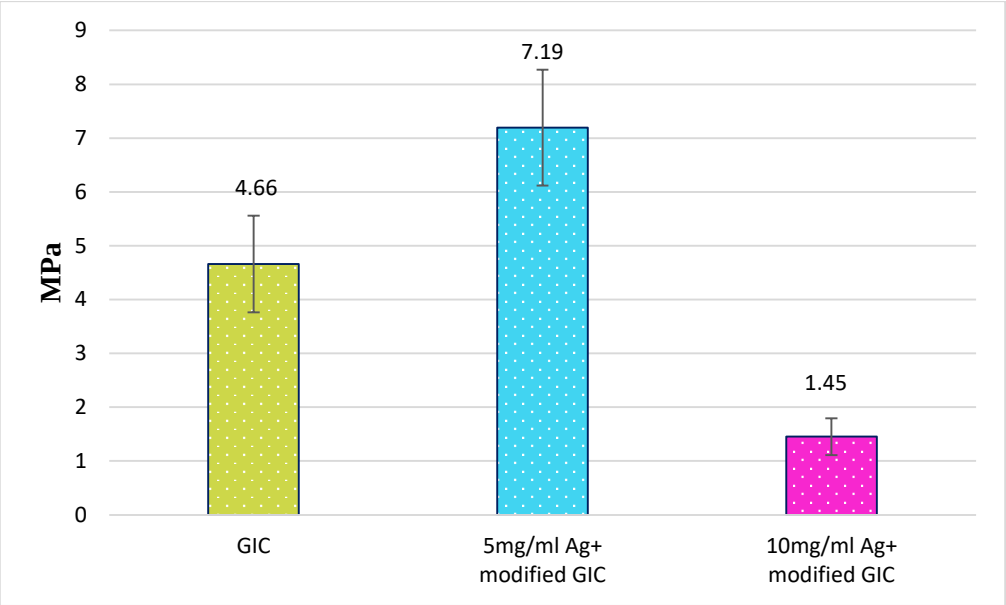


Figure 6.30 Shear Bond Strength of specimens of ChemFil Superior and silver modified ChemFil Superior (Mean±SD *n* =15).

The data conformed to a normal statistical distribution by using D'Agostino & Pearson omnibus normality test. Accordingly, an analysis of variance using Tukey comparison of means test was conducted and revealed highly statistically significant differences ($P < 0.0001$) between the values as summarised (Table 6.37) which shows that 5mg/ml silver addition to GIC significantly enhanced the shear bond strength of GIC while 10mg/ml silver addition adversely affect the bonding ability of GIC significantly.

Table 6.37 Tukey comparison of means of Shear bond strength values. Showing a highly significant difference between silver modified GIC and non-modified GIC ($p < 0.0001$).

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified GIC	< 0.0001
10mg/ml silver modified GIC – ChemFil Superior GIC	< 0.0001
5mg/ml silver modified GIC – ChemFil Superior GIC	< 0.0001

6.8.4 Hardness

6.8.4.1 Materials and Methods

- **Specimens**

  10 Silver modified specimens, 5 for each concentration of silver solution evaluated.

  5 Non-modified GIC specimens.

- **Specimen Testing**

A type D Shore Durometer (Shore Instrument and manufacturing Co, Jamaica, New York, USA) was used to measure this property (Figure 6.31). Prior to its use its calibration was checked against its supplied calibration and each sample was subjected to one indentation, yielding a Shore hardness value.

Figure 6.31 Shore hardness instrument.

For each state and material hardness values were expressed as a mean and standard deviation of five samples, each sample measured three times in three different locations (three reading from each specimen).

6.8.4.2 Results

Table 6.38 gives the raw data from the testing of surface hardness of specimens of GIC ChemFil Superior, ChemFil Superior with Silver additions. The units of measurement are shore hardness numbers. In addition, the mean and standard deviation of these observations following one week of storage in distilled water are presented in (Figure 6.32).

Table 6.38 Raw data from surface hardness testing of specimens; ChemFil Superior, ChemFil Superior with respective additions 5mg/ml and 10mg/ml PVA silver solutions.

Sample	GIC	5 mg/ml	10mg/ml PVA
1	50	75	65
2	45	79	60
3	45	85	60
4	43	85	61
5	40	75	67
6	43	85	70
7	45	85	68
8	50	90	70
9	50	90	60
10	49	85	65
11	49	93	70
12	50	87	70
13	50	97	70
14	49	95	75
15	45	85	70
Mean	46.86	86.06	66.73
SD	3.31	6.44	4.69

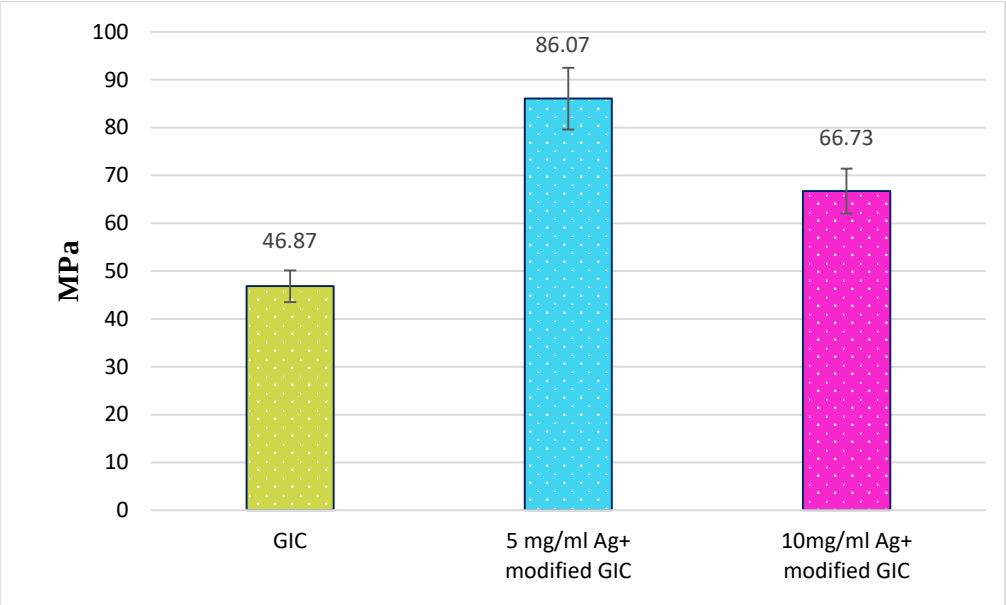


Figure 6.32 Surface hardness of ChemFil Superior and silver modified ChemFil Superior in Mega Pascal (MPa) (Mean±SD *n* =15).

In order to compare these results a normality test was performed using D'Agostino & Pearson omnibus normality test. Accordingly, an analysis of variance using a Tukey comparison of means test was conducted and revealed highly statistically significant difference ($P < 0.0001$) between the values as summarised in (Table 6.39) which shows that both silver formulations significantly enhanced the hardness of GIC.

Table 6.39 Tukey comparison of means shore Hardness values. Showing a highly significant difference between silver modified GIC and non-modified GIC (*p < 0.0001).*

Sample1-Sample2	Significance
10mg/ml silver modified GIC – 5mg/ml silver modified	< 0.0001
10mg/ml silver modified GIC – ChemFil Superior GIC	< 0.0001
5mg/ml silver modified GIC – ChemFil Superior GIC	< 0.0001

6.8.5 Discussion

The aim of this project was to investigate the potential to modify existing glass ionomer to enhance their antibacterial activity to improve the clinical success and decrease failure rates of restorations.

In order to establish if modification had an adverse effect on material properties, baseline property values were determined of the unmodified materials using known laboratory testing techniques.

In preparing the specimens for testing a clinically realistic mixing regime was followed. This was because it was envisaged that the material, and any additions to it, would be placed immediately into the oral cavity, so contrary to the manufacturer's instructions would be exposed to saliva. It was to part simulate this that no water impervious coatings were applied to the specimens prior to storage.

6.8.5.1 Testing Methods and Baseline Results

The comparison of physical properties of ChemFil Superior when it is modified to the previously published data is not directive, because materials and storage regimes differ. However, the values serve as a good source of indicative ball park figures to compare the present work against. In order to simplify the comparison of the baseline property values determined in the present study to those reported by others in the literature Table 6.40 summarises the values obtained by others.

Table 6.40 The properties of ChemFil Superior from other laboratory studies that examined some the same physical properties as the present study. VH: Vickers hardness; d: Day; m: Month; w: Week.

Authors	Materials	Compressive strength (MPa)	Hardness	storage time
(McKenzie et al., 2003)	ChemFil Superior	177.1±14.7/188.9±23.1/182.7±22.9/ 148.1±16.3 /192.0±24.5 /184.1±25.9	-	1 d/1 w/1 m/3 m/6 m/1 y
Pamir et al., 2005)	ChemFil Superior	148.30±9.63	49.89± (VH)	28 d
Türkün et al., 2008)	ChemFil Superior	211.1±14.1	57.23(VH)	1 d
Dowling & Fleming et al., 2009)	ChemFil Superior	132±18	-	1 d
Salem et al (Salem et al., 2016)	ChemFil Superior	50±19	36±7.4 (Shore H)	1 w

6.8.5.1.1 Compressive strength

Compressive strength was determined using an Instron universal testing machine at 1mm min^{-1}. As observed by others the ends of longitudinal compressive specimens must be lapped flat otherwise edge effects lead to premature failure of the specimen by facilitating crack initiation and propagation (Cho and Garant, 2000).The baseline results obtained in this study are in general agreement with other values published in the literature. Although values are lower than some of them while they are higher than Salem et al (2016), this is in all likelihood due to both the mixing and storage regime as stated in the introductory paragraph (6.8.5.1).

Compressive strength is considered to be of relevance to clinical function (Cho and Garant, 1989). Mastication applies compressive forces as tested here by the compressive strength (Freeman and ten Cate, 1971).

6.8.5.1.2 Shore Hardness

In this work Shore hardness was determined. This test was selected over the more usually performed Vickers hardens because of multiple failures to obtain a consistent result with Vickers hardness testing machine, in addition to loss of most of reading stored on that machine because errors with the machine's software therefore the decision was to adopt a relative ease of penetration test, such as afforded by the Shore tester, to enable a hardness assessment to be made. A downside of this approach is that the results presented here cannot be compared to other literature values for no other workers have employed this test for glass ionomers.

6.8.5.1.3 Adhesive Shear Bond Strength to Dentine

As mentioned earlier glass-ionomer has the ability to bind chemically to tooth tissues, in this project an assessment of the bond of the glass ionomer to tooth substance was made. Self-adhesion is a unique property of the glass ionomers studied here. It is considered important to the proposed application of the modified materials of this work for it will provide a marginal seal and thus, if durable prevent the ingress of bacteria and of recurrent caries besides posing antibacterial activity. Its determination is however, controversial in respect of testing method and tooth substrate used.

Human teeth were selected and used in this study rather than bovine teeth for the sake of more specific results, an examination of the baseline bond strengths of the materials in unmodified form (Table 6.36) the values obtained.

6.8.5.2 The Physical Properties of the Glass-Ionomer Cements Following Silver Additions

All tested properties improved significantly, compared to the baseline values, for the samples of ChemFil Superior to which 5mg/ml silver solution was added. As regards additions of 10mg/ml PVA similar improvements were observed for all properties except shear bond strength Figures (6.28), (6.30) and (6.32).

In relation to shear bond strength, 10mg/ml with PVA compromised the bond strength significantly although specimens were sticky to hand after setting. Perhaps this is due to the presence of PVA which in a way prevents the chemical linkage with calcium of the tooth surface to occurring, because 5mg/ml silver solution addition which did not include PVA component enhanced the bond strength significantly. The enhanced bond strength with 5mg/ml may be due to the acidity of the solution. It has been observed that the pH of a setting conventional glass ionomer (ChemFil) changes with time commencing around 2.2 and increasing to 6.2 after1440 minutes (Smith and Ruse, 1986). 5mg/ml contains 0.5M citric acid which may roughen the surface of the tooth in order to promote micromechanical attachment beside the chemical bond of glass-ionomer material.

Metal reinforced glass ionomer cements (e.g. Ketac Silver, Espe) which contain silver-tin alloy fibres or flakes being sintered to the glass particles (Mount, 1998; Nicholson, 1997) were developed to improve the mechanical properties of the cements and increase their wear resistance (Meffert, 1996; Walls, 1987; Williams J A, 1992). Due to the amalgam-like optical performance, metal-reinforced materials showed enormous commercial success and were consequently used as posterior materials for primary dentition as well (Krämer N, 1996). However, a clinical study by Kilpatrick (Kilpatrick, 1993), demonstrated that Ketac Silver was inferior to the conventional non-metal-reinforced glass ionomer cement Ketac Fil in Class II cavities. Clinical reports dealing with non-modified glass ionomers in Class II restorations of primary molars show

unfavourable results in general (Randall and Wilson, 1999, Holst, 1996) which lead to the development of glass ionomer cements focussed on a higher powder-to-liquid ratio, a lower water content, and smaller glass particles leading to high viscosity glass ionomer cements (nonmetal-reinforced: Fuji IX, Ketac Molar; metal-reinforced: Hi Dense) which should be packable like amalgam and reveal enhanced strength characteristics (Guggenberger, 1998; Holst, 1996; Williams, 1992). However, 5mg/ml enhanced significantly all the physical properties tested. In addition, it can be assumed to have an enhanced radioapcity due to silver content. Consequently, 5mg/ml silver citrate citric acid complex could be considered a promising additive to glass ionomer cement hence it enhanced both its antibacterial and physical properties.

6.9 Summary of Major findings

- The three silver citrate citric acid complex solutions showed a potent antibacterial activity against early coloniser model species in solution, 13mg/ml silver solution had the highest antibacterial activity followed by 5mg/ml silver solution and lastly 10mg/ml silver solution. Part of the killing activity of 13mg/ml was related to its high acidity because it contained 2.7M citric acid which was needed to solubilise silver citrate during its formation method.
- 13mg/ml silver solution addition to GIC failed to form stable formulation because of the high acidity of the silver solution which adversely affected the setting reaction of GIC.
- 5mg/ml and 10mg/ml silver addition to GIC significantly decreased the colonisation of the early coloniser species after 24Hrs aerobic incubation as was proven from CFUs and MTT results. However, 10mg/ml silver enhanced GIC enhanced the colonisation of the three species significantly after 24Hrs and 48Hrs anaerobic incubation.
- 5mg/ml enhanced GIC disks showed a significant decrease in colonisation after 48Hrs anaerobic incubation.
- 10mg/ml silver citrate citric acid complex solution because of its PVA constituent, adversely affected the antibacterial properties and the shear bond strength of GIC when it was added to it.
- 5mg/ml silver enhanced GIC showed a significant enhanced compressive strength, hardness and shear bond strength.

Chapter 7 OVERALL DISCUSSION AND CONCLUSIONS

Biofilm models can be difficult to compare due to the differences in biofilm formation times, different growth media and varying bacterial species used in different situations. However BMM is a widely used artificial saliva, DMM is still considered a rich media despite the slightly slower growth of early coloniser model species compared to CB as it is demonstrated in this study or described by Wong et al (Wong and Sissons, 2001) in comparison to BMM. DMM usage has an integral role in *in vitro* biofilm modelling because it is a chemically defined formula in which known changes can be made to individual components, for example, to study factors regulating plaque growth and mineralisation. Consequently, it is possible to manipulate amino acids, vitamin and ion components of the medium to determine their effect on plaque bacterial growth, composition and properties, and pathogenicity. It contains commercially available components that simulate relevant constituents of saliva. In addition the presence of mucin in DMM plays a critical role in acquired pellicle formation which is considered to be the first step in biofilm formation (Gibbins et al., 2014, Hannig and Joiner, 2006).

The early coloniser biofilm model could be considered as a modified Batch culture biofilm model. This model was adapted to create a liquid shear force onto the formed biofilm through a consistent and reproducible five washing steps protocol. In this study five washing series efficiently reduced the amount of the loosely attached bacterial cells colonising GIC surfaces rendering an intimately attached biofilm with stronger adhesion to GIC surfaces, this was confirmed by SEM images, DNA quantification and CFUs assay.

Although some models have been adapted to create a liquid shear force by dipping the biofilms in saline or other liquid 3 times a day during biofilm formation (Guggenheim et al., 2004), that process is quiet complicated compared to the five washing series. Despite this neither method simulates the exact sheer force of saliva. The washing series is simpler and could be adapted to other systems. Future work is needed to compare the intimately attached biofilms remaining after the washing series with biofilms resulting from AMM or flow cell studies, because these latter methods are claimed to have the nearest system to oral environment by simulation of saliva shear force.

The most significant advantages of batch models are their ability to offer means of comparing multiple test compounds or conditions simultaneously; they only require small amounts of reagents and are convenient, reproducible, and economical to use (Coenye and Nelis, 2010).

Early coloniser biofilm represent a simple reproducible and effective batch model; compared to microcosm biofilm models which used saliva as an inoculum (Rudney et al., 2012).

One of the most commonly used batch biofilm models is the Zürich biofilm model which uses six microbial species (*S. oralis, S. sobrinus, A. naeslundii, V. dispar, F. nucleatum, and Candida albicans*) (Guggenheim et al., 2004). Using fluorescently labelled antibodies and confocal laser scanning microscopy (CLSM), this model allows the interspecies associations to be studied with respect to biofilm formation and how macromolecules of different sizes can penetrate the biofilm *in vitro*. This model and its variants where they used smaller numbers of bacteria have been used extensively to evaluate the effect of different substances in the biofilm formation process. However, the model set up is very complex and it was done only under anaerobic conditions without consistent washing steps and without chemically defined or mucin containing artificial

saliva recipe. In comparison, early coloniser model is simpler in design with the added value of mucin containing and chemically defined DMM. Although they both have some species in common, the consortium of this project has more species implicated in dental caries and oral diseases. SEM was used rather than CLSM where the visualisation is indirect and dependent upon fluoresce of labelled antibodies.

None of the previous biofilm designs considered investigating the growth and survival of species around the tested materials; they were only concerned with their attachment over material surfaces. Because a batch culture is closed system ignoring this will result in underestimating the effect of those materials on the ecology of the biofilm formed and less understanding of the mechanism of action of several antibacterial additives which have the ability to be released in the system and decrease bacterial growth. In this model a detailed investigation of the planktonic growth around GIC disks using CFUs and MTT assays was done and revealed the possible effect of released fluoride and silver ions into the system.

In addition, most of the previous studies they used 24 well plates which offers less volume than 6 well plates i.e. each well of 24 well plate has a total volume of 3.4 mL while each well of 6 well plate has a 16.8 mL total volume. The 6 well plates allowed 5ml of culture medium with a sufficient distance from the lid that is important for oxygen exchange, therefore more nutrients and more dilution of any released antibacterial substance. In addition, this volume allowed the future adaptation of larger materials or teeth compared to 24 well plate which in that instance the material/volume ratio will be an important issue.

The early coloniser model proved its efficacy by assessing the antibacterial activity of additive to GIC in detail; 13mg/ml silver solution showed the highest killing potency against the early coloniser model in solution (MIC/MBC), but once added to GIC it failed

to form a stable formulation and that was probably due to the high acidity of silver solution. Aerobically and anaerobically fluoride release from GIC seems to have an effect on the growth of early coloniser model species especially anaerobically after 48Hrs. This in a way may explain the debatable effect of fluoride release effect; bearing in mind the dilution of fluoride released in 5ml of solution despite it didn't give any significant killing in NaF form anaerobically. However, 10mg/ml silver solution showed a killing activity in solution in addition its addition to GIC significantly reduced the growth and colonisation of early coloniser model species aerobically; anaerobically its addition to GIC significantly enhanced the growth and attachment of model species around and on GIC disks.

The early coloniser model is considered the foundation for the whole biofilm model, which is modelled in a structured manner depending on the known dental plaque formation mechanism; it starts with early coloniser's attachment to acquired pellicle, then the addition of late colonisers and bridging bacterial cells like *F. nucleatum* later in future work.

Silver was chosen in this study because of its extraordinary bacteriostatic and bactericidal properties. The silver ion exhibits broad-spectrum biocidal activity toward many different bacteria, fungi, and viruses and is accepted to be the active component in silver-based antimicrobials (Panacek et al., 2006).

Most of the past research has concentrated on Nano metric silver particles (AgNPs) prepared by a variety of synthetic methods and several formulations and they considered an effective antimicrobial agents (Alt et al., 2004, Aymonier et al., 2002, Baker et al., 2005, Melaiye et al., 2005, Sondi and Salopek-Sondi, 2004). But their formation is technique sensitive and a great effort has been made to have a stable formulation, instability of silver in solution affects their durability and clinical applications in dentistry

because they need to have a reasonable storage time where they show an excellent stability in solution form. In addition, AgNPs are commercially available but very expensive as mentioned earlier.

Silver citrate/citric acid complexes proved their potent antimicrobial activity. Though their preparation is less technique sensitive and they can be easily prepared in labs especially at concentrations of around 5mg/ml.

The solutions of silver citrate/citric acid had exhibited a relatively good stability; 10mg/ml was the most stable formulation; it did not show any recrystallisation for over 6 months which might be related to the presence of PVA stabiliser, in addition 5 mg/ml showed good stability during a 6 month period. In other words, under these conditions, the concentration of citric acid was sufficient to allow the existence of silver citrate complexes in solution. 13mg/ml solution showed recrystallisation within 3 months of formation, however that didn't significantly affect its antibacterial activity within the experimental three months. Compared to other studies, 22mg/ml solution failed to show stability over 3 days only, despite others claiming it was stable for 13 weeks (Djokic, 2008). In both studies the dilution of 22 mg/ml solution to reach 13mg/ml exhibited better stability. It seems that the silver citrate/citric acid complexed solutions are quite stable for a relatively long period of time, when the concentration of Ag^{+} ion is less than 15 g/L and the concentration of citric acid is more than 3 mol/L (576 g/L). These solutions have not exhibited changes in colour and, also, a formation of precipitate did not occur. Contrarily, these solutions were clear and colourless for more than 6 months, suggesting as well a good stability when exposed to light.

Despite 13mg/ml silver failing to form a stable formulation with GIC, potential antibacterial activity could be tested if incorporated with other materials like implant surfaces. Also, 10mg/ml silver solution addition to GIC failed to enhance its antibacterial

activity anaerobically but its antibacterial activity could be tested with other dental restorative materials e.g. RMGIC and dental implants.

Glass ionomer cement with the addition of 5mg/ml silver solution has demonstrated enhanced compressive, hardness and shear bond strengths, in addition it can be assumed to have an enhanced radioapcity because of the presence of silver ions. However, the addition of 10mg/ml silver solution adversely affected the shear bond strength of GIC to dentine consequently affecting its clinical application in the oral cavity.

Within the parameters of this study, 5mg/ml silver citrate citric acid solution showed a potent antibacterial activity in solution especially after 24Hrs. Its addition to GIC significantly enhanced its physical properties.

In Conclusion;

- The Early coloniser biofilm model is a simplified, shear force adapted and reproducible batch *in vitro* model. In addition, it is considered the first step in a logical following multistep *in vitro* model when other consortium species are added in the future work.
- The Early coloniser model proved its efficacy in assessing the antibacterial activity of conventional GIC in its basic formula or silver enhanced form.
- The Early coloniser model has the potential to test materials with different sizes and forms because of the well size provided by 6 well plate vessel.
- Fluoride release from conventional GIC proved to affect the colonisation and growth of early coloniser species within 24-48Hrs aerobically and anaerobically although it was not statistically significant.
- Silver citrate/citric acid complexes showed a good stability over 6 months' period of time, therefore affecting their future clinical applications. In addition, they are

considered cheap lab made silver solution which are relatively easy to make compared to AgNPs.

- 10mg/ml silver addition to glass ionomer cement adversely affect the antibacterial properties of GIC anaerobically because of PVA addition to the silver solution. In addition, it adversely affects the adhesive properties of GIC to tooth tissues.
- The antibacterial activity and physical properties of conventional GIC were successfully enhanced with the addition of 5mg/ml silver citrate citric acid complexes.

Chapter 8 FUTURE WORK

- To continue with the next step towards building up the whole biofilm through adding late colonisers and bridging microorganism's e.g. *F. nucleatum* to investigate their effect on the biofilm formed.
- To continue in developing qPCR primers to selectively quantify early coloniser species in mixed community.
- Investigate the effect of silver solutions addition to other dental materials e.g. RMGI and implant surfaces.
- Assess fluoride and silver release in DMM with and without bacterial community using ion electrodes.
- Investigate the potential effect of PVA on bacterial growth.
- Test silver toxicity effect against human cell lines.
- Use either the Flow cell model or AMM to form an early coloniser model and compare results with the modified Batch early coloniser model.

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Chapter 10 APPENDICES

10.1 Appendix 1. PCR analysis

10.1.1 DNA sequence of obtained from amplification of *gtfB* gene of *S. oralis* samples.

```
S oralis GTFb f   1                                                                 0
S oralis GTFb r   1 CTTTCGGGTGGCTTGGCAGCAAACTATCCGTTYGCAACTATAGACCCAAACGTGGGTATT  60


S oralis GTFb f   1                                                  TAAAAAAACA  10
S oralis GTFb r  61 GTAGAAGTGCCAGATGCAAGACTACTTAAATTAGAAGAAATGGTTCAACCTAAAAAAACA 120
                                                                     **********

S oralis GTFb f  11 TTACCTACTACWTTTGAATTWWCRSATATTGCCGGAATTGTKMAWGGTGCTTCKAAAGGT  70
S oralis GTFb r 121 TTACCTACTACATTTGAATTTACAGATATTGCCGGAATTGTTAAAGGTGCTTYTAAAGGT 180
                    *********** ********  *  ****************  * *******  ******

S oralis GTFb f  71 GAAGGTTTARGTAMTAAWTTCTTATCACATATTAAARAASTAKATGCMATTTGKCAGGTT 130
S oralis GTFb r 181 GAAGGTTTAGGTAATAAATTCTTATCACATATTAGAGAAGTAGATGCAATTTGTCAGGTT 240
                    ********* *** *** ***************** * ** ** **** ***** ******

S oralis GTFb f 131 GTTCKTGCTTTTGACRATGATAATGTAACACATGTAGCTGGTCKAGTTGATCCAATTGAT 190
S oralis GTFb r 241 GTTCGTGCTTTTGACGATGATAATGTAACACATGTAGCTGGTCGAGTTGATCCAATTGAT 300
                    **** ********** *************************** ****************

S oralis GTFb f 191 GACATCKAARTTATCAATATGGAATTASTACTTGCARACTTAKAATCARWTGAAAAMCSC 250
S oralis GTFb r 301 GACATCGAAGTTATCAATATGGAATTAGTACTTGCAGACTTAGAATCAGTTGAAAAACGC 360
                    ****** ** ***************** ******** ***** *****  ****** * *

S oralis GTFb f 251 TTACCTARAAWTGAAAAMTTGGCACRTCAAAAASATAAGACTGCGGAAATGGAAKTGCGT 310
S oralis GTFb r 361 TTACCTAGAATTGAAAAATTGGCACGTCAAAAARATAAGACTGCGGAAATGGAAGTGSGT 420
                    ******* ** ****** ******* ******* ******************** ** **

S oralis GTFb f 311 ATTTTAACAACTATTAAWKAARCTTTRGAAAATGSYMAMCCCGCTCKTASTATTGACTTT 370
S oralis GTFb r 421 ATTTTAACAACTATTAAAGAAGCTTTAGAAAATGGTAAACCCGCTCGTAGTATTGA     476
                    *****************  ** **** *******   * ******* ** ******

S oralis GTFb f 371 AATGAARAARATCARARATGGGTGAATC 398
S oralis GTFb r 477                             476
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CTTTCGGGTGGCTTGGCAGCAAACTATCCGTTYGCAACTATAGACCCAAACGTGGGTATTGTAGAAGTGCCAGATGCAAGACTACTTA
AATTAGAAGAAATGGTTCAACCTAAAAAAACATTACCTACTACATTTGAATTTACAGATATTGCCGGAATTGTTAAAGGTGCTTCTAA
AGGTGAAGGTTTAGGTAATAAATTCTTATCACATATTARAGAAGTAGATGCAATTTGTCAGGTTGTTCGTGCTTTTGACGATGATAAT
GTAACACATGTAGCTGGTCGAGTTGATCCAATTGATGACATCGAAGTTATCAATATGGAATTAGTACTTGCAGACTTAGAATCAGTTG
AAAAACGCTTACCTAGAATTGAAAAATTGGCACGTCAAAAAVATAAGACTGCGGAAATGGAAGTGCGTATTTTAACAACTATTAAAGA
AGCTTTAGAAAATGGTAAACCCGCTCGTAGTATTGACTTTAATGAARAARATCARARATGGGTGAATC
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10.1.2 DNA sequence of obtained from amplification of *rplF* gene from *N. subflava* samples.

```
Neisseria rplB    1                                                            0
Neisseria rplB    1 GTGACTGTTCCCGCTGGTGTAGAAGTAAAATTCGGAACAGATGCATTGGTTATCAAAGGT  60


Neisseria rplB    1      CGGTGAATTGTCTTTTCCTTTGCATTCCGATGTTGCTATTGAACTGAACGATGGT  55
Neisseria rplB   61 AAAAACGGTGAATTGTCTTTTCCTTTGCATTCCGATGTTGCTATTGAACTGAACGATGGT 120
                    ********************************************************


Neisseria rplB   56 AAATTGACTTTTGCTGCAAAAAATGACAGTAAACAGGCTAATGCTATGTCTGGTACTGCT 115
Neisseria rplB  121 AAATTGACTTTTGCTGCAAAAAATGACAGTAAACAGGCTAATGCTATGTCTGGTACTGCT 180
                    ************************************************************


Neisseria rplB  116 CGTGCATTGGTTAATAACATGGTTAAGGGAGTTTCTGAAGGTTTTGAGAAAAAACTTCAA 175
Neisseria rplB  181 CGTGCATTGGTTAATAACATGGTTAAGGGAGTTTCTGAAGGTTTTGAGAAAAAACTTCAA 240
                    ************************************************************


Neisseria rplB  176 TTGATTGGTGTGGGTTATCGTGCCCAAGCTCAAGGTAAAGTTTTGAACCTGTCTTTGGGT 235
Neisseria rplB  241 TTGATTGGTGTGGGTTATCGTGCCCAAGCTCAAGGTAAAGTTTTGAACCTGTCTTTGGGT 300
                    ************************************************************


Neisseria rplB  236 TTCTCTCATCCAATCGTATATGAAATGCCTGAAGGTGTTTCCGTTCAAACTCCTAGCCAA 295
Neisseria rplB  301 TTCTCTCATCCAATCGTATATGAAATGCCTGAAGGTGTTTCCGTTCAAACTCCTAGCCAA 360
                    ************************************************************


Neisseria rplB  296 ACAGAAATCGTTTTGACTGGTGCAGATAAACAAGTGGTTGGCCAAGTCGCTGCTGAAATT 355
Neisseria rplB  361 ACAGAAATCG                                                  370
                    **********


Neisseria rplB  356 CGTGCRTTCCG 366
Neisseria rplB  371             370
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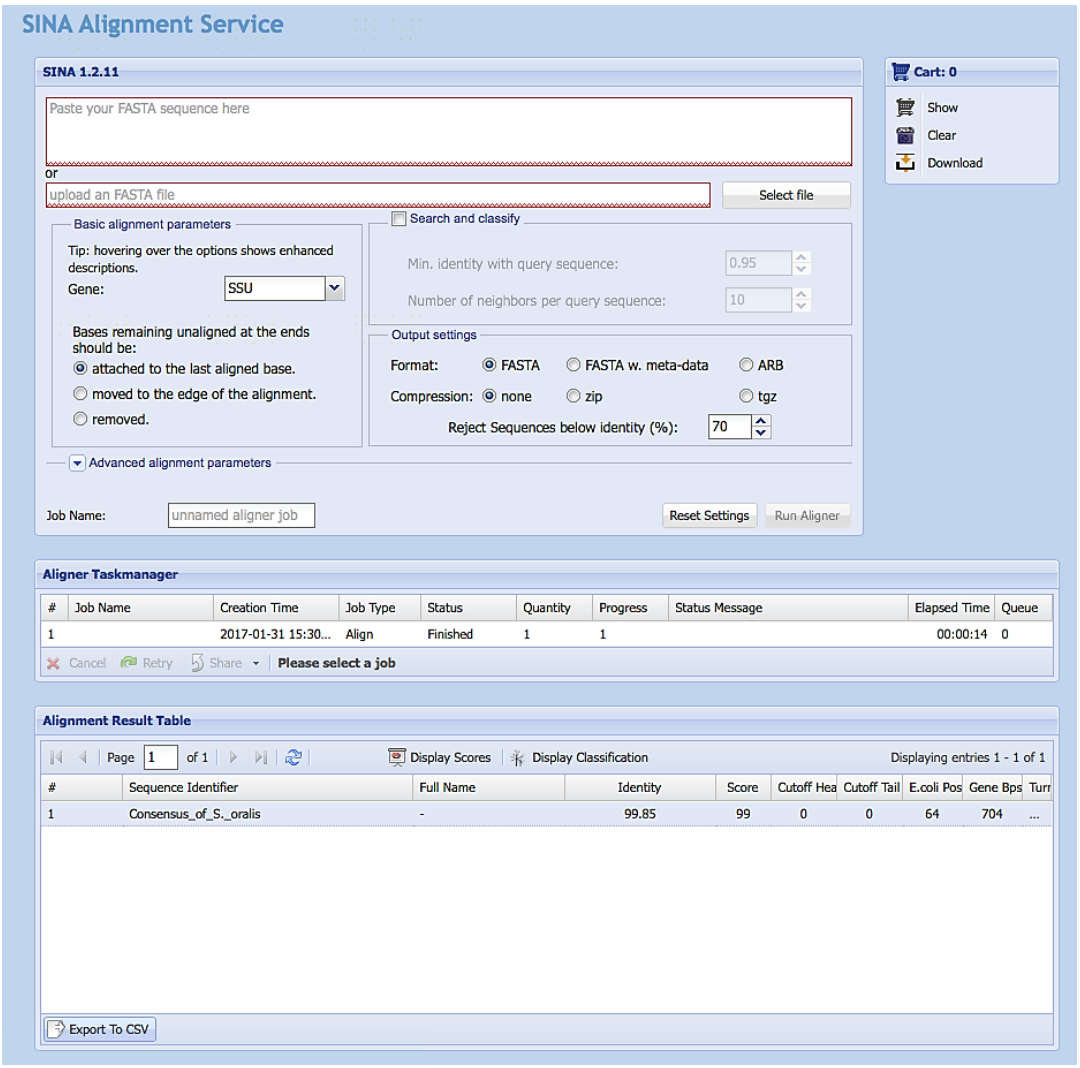
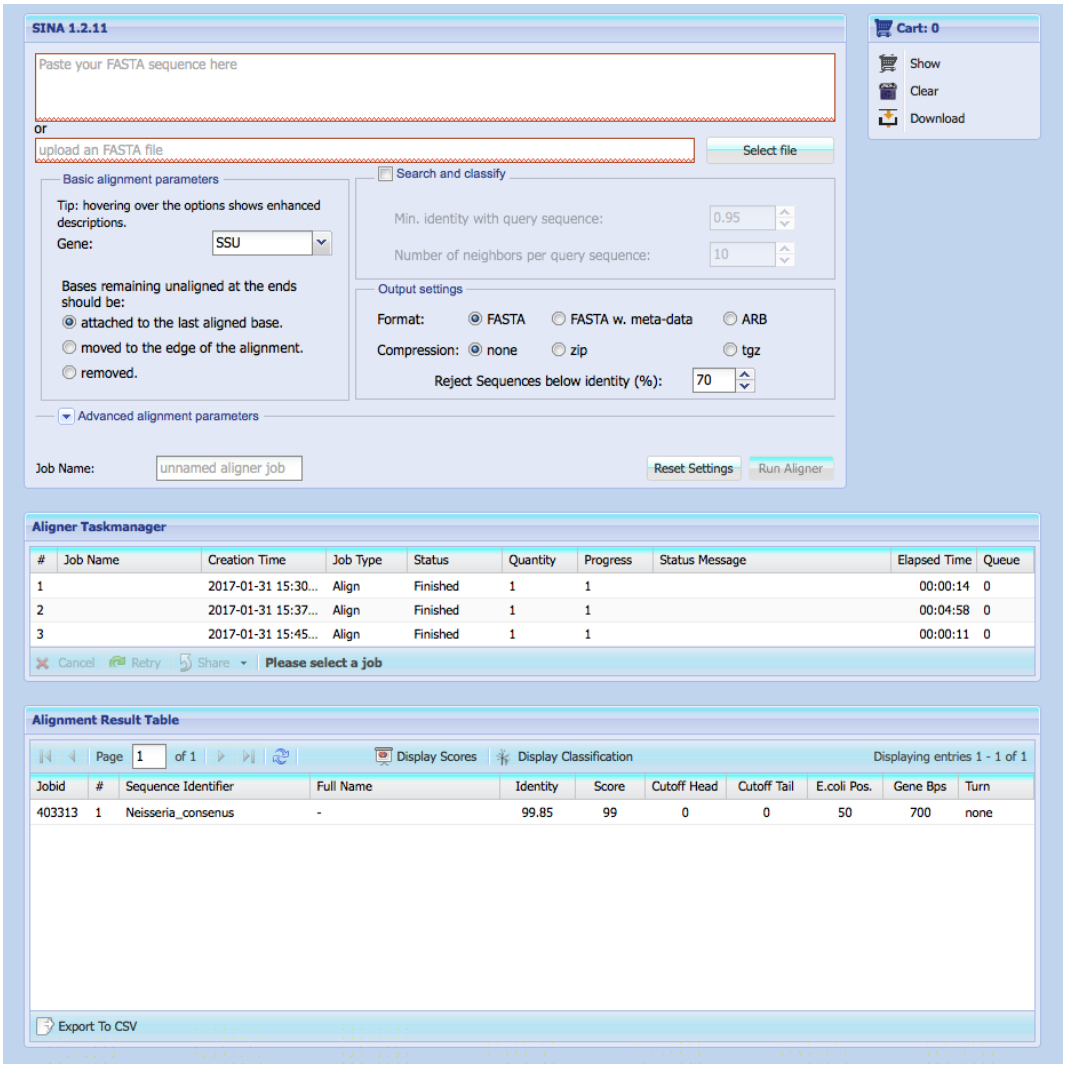
GTGACTGTTCCCGCTGGTGTAGAAGTAAAATTCGGAACAGATGCATTGGTTATCAAAGGTAAAAACGGTGAATTGTCTTTTCCTTTGC
ATTCCGATGTTGCTATTGAACTGAACGATGGTAAATTGACTTTTGCTGCAAAAAATGACAGTAAACAGGCTAATGCTATGTCTGGTAC
TGCTCGTGCATTGGTTAATAACATGGTTAAGGGAGTTTCTGAAGGTTTTGAGAAAAAACTTCAATTGATTGGTGTGGGTTATCGTGCC
CAAGCTCAAGGTAAAGTTTTGAACCTGTCTTTGGGTTTCTCTCATCCAATCGTATATGAAATGCCTGAAGGTGTTTCCGTTCAAACTC
CTAGCCAAACAGAAATCGTTTTGACTGGTGCAGATAAACAAGTGGTTGGCCAAGTCGCTGCTGAAATTCGTGCRTTCCG
```

10.1.3 *S. oralis* identify confirmation.



10.1.4 *S. mutans* Identity confirmation.

10.1.5 *N. subflava* identity confirmation.



10.1.6 *Gtf* gene sequence in *S. oralis* aligned with *rgg* gene sequence.

```
S oralis SOR_13    1 ATGATGGAGAAAAAGATTCATTATAAGATGCATAAAGTTAAGAAAAACTGGGTAGCCATT   60
Untitled           1 ATGATGGAGAAAAAGATTCATTATAAGATGCATAAAGTTAAGAAAAACTGGGTAGCCATT   60
                     ************************************************************

S oralis SOR_13   61 GCTTTGACTACCTTGGCCCTTATTGTAGCACCAAAGGTACTTGGTCTAGAACCAGGCGTT  120
Untitled          61 GCTTTGACTACCTTGGCCCTTATTGTAGCACCAAAGGTACTTGGTCTAGAACCAGGCGTT  120
                     ************************************************************

S oralis SOR_13  121 GTCCATGCGGATGATGTAAAGCAGGTTGTAGTTCAAGAACCTGCTACAGCTCAGACTAGT  180
Untitled         121 GTCCATGCGGATGATGTAAAGCAGGTTGTAGTTCAAGAACCTGCTACAGCTCAGACTAGT  180
                     ************************************************************

S oralis SOR_13  181 GGTCCGGGTCAGCAAACTCCAGCCCAAGCTAAAATAGCATCTGAGCAAGAAGCAGAAAAA  240
Untitled         181 GGTCCGGGTCAGCAAACTCCAGCCCAAGCTAAAATAGCATCTGAGCAAGAAGCAGAAAAA  240
                     ************************************************************

S oralis SOR_13  241 GCAACCCCTGCAGACAAGGTGACAGGCGATGCTGCTGCTAGTGATAAGTCTGCTAAACCA  300
Untitled         241 GCAACCCCTGCAGACAAGGTGACAGGCGATGCTGCTGCTAGTGATAAGTCTGCTAAACCA  300
                     ************************************************************

S oralis SOR_13  301 GCAGAAAATACGGAAGCAACAGTTCAAACCAATGCTCAAGAGCCTGCTAAACCAGCAGAT  360
Untitled         301 GCAGAAAATACGGAAGCAACAGTTCAAACCAATGCTCAAGAGCCTGCTAAACCAGCAGAT  360
                     ************************************************************

S oralis SOR_13  361 ACGAAAGAAGCATCTACAGAAAAGGCTGCTGTTGCTGAAGAAGTTAAAGCTGCTAATGCA  420
Untitled         361 ACGAAAGAAGCATCTACAGAAAAGGCTGCTGTTGCTGAAGAAGTTAAAGCTGCTAATGCA  420
                     ************************************************************

S oralis SOR_13  421 ATCACAGAAACTCCTAAAACAGAAGTAGCAGACCAGGATAAACAAGCAAGGCCAACAACT  480
Untitled         421 ATCACAGAAACTCCTAAAACAGAAGTAGCAGACCAGGATAAACAAGCAAGGCCAACAACT  480
                     ************************************************************

S oralis SOR_13  481 GCCCAAGACCAAGAAGGCGACAAACGAGAAAAATCGGCTATTGAAGACAAGATTGTTGCA  540
Untitled         481 GCCCAAGACCAAGAAGGCGACAAACGAGAAAAATCGGCTATTGAAGACAAGATTGTTGCA  540
                     ************************************************************

S oralis SOR_13  541 AATCCAAAGGTTGCAAAGAAAGATCGCTTGCCCGAACCTGCTCAAAAACAAGGAGCAGTA  600
Untitled         541 AATCCAAAGGTTGCAAAGAAAGATCGCTTGCCCGAACCTGCTCAAAAACAAGGAGCAGTA  600
                     ************************************************************

S oralis SOR_13  601 GCTGAAAGAATGGTAGCAGATCAGGCTCAACCTGCACCTGTAAATGCTGACCATGATGAT  660
Untitled         601 GCTGAAAGAATGGTAGCAGATCAGGCTCAACCTGCACCTGTAAATGCTGACCATGATGAT  660
                     ************************************************************

S oralis SOR_13  661 GATGTCCTATCTCATATTAAGACCATTGATGGTAAAAATTACTATGTTCAGGACGATGGT  720
Untitled         661 GATGTCCTATCTCATATTAAGACCATTGATGGTAAAAATTACTATGTTCAGGACGATGGT  720
                     ************************************************************

S oralis SOR_13  721 ACAGTTAAAAAGAACTTTGCAGTTGAACTTAATGGGAGAATACTTTATTTTGATGCAGAA  780
Untitled         721 ACAGTTAAAAAGAACTTTGCAGTTGAACTTAATGGGAGAATACTTTATTTTGATGCAGAA  780
                     ************************************************************

S oralis SOR_13  781 ACGGGTGCCTTAGTTGATTCAAATGAATATCAGTTCCAACAAGGAACCAGCAGTCTCAAT  840
Untitled         781 ACGGGTGCCTTAGTTGATTCAAATGAATATCAGTTCCAACAAGGAACCAGCAGTCTCAAT  840
                     ************************************************************

S oralis SOR_13  841 AATGAATTCTCCCAAAAGAATGCTTTCTATGGTACGACTGACAAGGATATTGAAACTGTA  900
Untitled         841 AATGAATTCTCCCAAAAGAATGCTTTCTATGGTACGACTGACAAGGATATTGAAACTGTA  900
                     ************************************************************

S oralis SOR_13  901 GACGGTTATTTGACAGCAGATAGCTGGTATCGTCCAAAGTTCATCTTAAAAGATGGAAAA  960
Untitled         901 GACGGTTATTTGACAGCAGATAGCTGGTATCGTCCAAAGTTCATCTTAAAAGATGGAAAA  960
                     ************************************************************

S oralis SOR_13  961 ACATGGACGGCATCGACAGAAACAGACTTACGTCCTCTTTTGATGGCTTGGTGGCCTGAT 1020
Untitled         961 ACATGGACGGCATCGACAGAAACAGACTTACGTCCTCTTTTGATGGCTTGGTGGCCTGAT 1020
                     ************************************************************

S oralis SOR_13 1021 AAACGTACTCAGATTAATTACCTCAACTATATGAACCAGCAAGGTCTGGGAGCAGGTGCT 1080
Untitled        1021 AAACGTACTCAGATTAATTACCTCAACTATATGAACCAGCAAGGTCTGGGAGCAGGTGCT 1080
                     ************************************************************
```

```
S oralis SOR_13 1081 TTTGAAAACAAAGTGGAACAAGCTCTCCTGACAGGTGCTTCTCAGCAGGTTCAGCGCAAA 1140
Untitled        1081 TTTGAAAACAAAGTGGAACAAGCTCTCCTGACAGGTGCTTCTCAGCAGGTTCAGCGCAAA 1140
                     ************************************************************

S oralis SOR_13 1141 ATTGAGGAAAAAATTGGTAAAGAAGGCGATACCAAATGGTTGAGAACGCTGATGGGTGCC 1200
Untitled        1141 ATTGAGGAAAAAATTGGTAAAGAAGGCGATACCAAATGGTTGAGAACGCTGATGGGTGCC 1200
                     ************************************************************

S oralis SOR_13 1201 TTTGTCAAAACCCAGCCAAACTGGAATATCAAGTCAGAGTCTGAAACAACTGGTACTAAA 1260
Untitled        1201 TTTGTCAAAACCCAGCCAAACTGGAATATCAAGTCAGAGTCTGAAACAACTGGTACTAAA 1260
                     ************************************************************

S oralis SOR_13 1261 AAGGACCACTTGCAGGGTGGAGCTTTGCTTTATACTAATAATGAGAAGAGCTCTCATGCT 1320
Untitled        1261 AAGGACCACTTGCAGGGTGGAGCTTTGCTTTATACTAATAATGAGAAGAGCTCTCATGCT 1320
                     ************************************************************

S oralis SOR_13 1321 GACTCTAAGTTCCGTCTGCTTAACCGTACCCCTACTAGTCAAACAGGTAAACCTAAGTAC 1380
Untitled        1321 GACTCTAAGTTCCGTCTGCTTAACCGTACCCCTACTAGTCAAACAGGTAAA          1371
                     ************************************************

S oralis SOR_13 1381 TTCATTGACAAGTCAAACGGTGGTTATGAGTTCTTGCTCGCTAACGACTTTGACAACTCT 1440
Untitled        1372                                                           1371
```

10.1.7 *S. oralis rgg* gene sequence aligned with *S. mutans rgg* sequence.

```
Oralis ORF2 rgg   1                    ATGTTGGAAACTTTCGGAAAAATATTCAAAGTCATTAG-A  39
Mutans ORF2 rgg   1 ATGGCAATTGCAGGTTTAGTTTTTCAAAAGTTACTCGTTTTATTTTTGCTGATGTTGGCA  60
* *   **  *    *    ** **    *   ** * *

Oralis ORF2 rgg  40 GAATCAAAAAAAAATGTCCCTGAAAGAGGTGGCTGCTGGTGATATTT--CCGTGGCTCA--  95
Mutans ORF2 rgg  61 GGATTATTTTTAGCGCAGCGGCAGATTTTAACCAATGAGGTTACTTTACAGCTATCTAAA 120
* ** *     *  *   * * *     *  *   **  * ** **  * *      *

Oralis ORF2 rgg  96 --GCTATCCCGTTTTGA-ACGGGGAGTCAATGGGAT-CACACTTGATTCTTTTTATTGTT 151
Mutans ORF2 rgg 121 CTGCTGACGCGTTTTGTTGCACCCAGTCTTTTTATTTCGTCTTTTATTTATCAAGCATTT 180
***  * *******   *    ****  *    * *    ** ***  *       **

Oralis ORF2 rgg 152 GTTTA-AAAAATATGGCTGTTTCCCTAGAGGAGTTTCAGTATGTTTACCATAATTACATT 210
Mutans ORF2 rgg 181 ACTTGGAAAAAAAATGATTTTATTGTTTAGTATGATTGGGGCTGCTTTTTTTCTGCTTATT 240
**  ***** ***  * * *   *      * **  *  ** **    *      ***

Oralis ORF2 rgg 211 GATTCAGATGATGT-GCTGTTCTCAAAAAAAGTAGCTGATGCATATCAGGAAAACAATGT 269
Mutans ORF2 rgg 241 ACTCGGATTGTTATTGTTTCATTTTTGCTTCCTAAAGAAAGGGCAACGGATAAATA-TGC 299
*     ** * * * *    *         **    * *   * * *  *** * **

Oralis ORF2 rgg 270 TGTCAAGCTCCAAAATATTTTGTCAAGCTCAGAAGCTTTGACCGA-ACAGTTTCCTGAGA 328
Mutans ORF2 rgg 300 TGTTCTTTTTGCTAACGTTGGTTTCATGGGAACTCCTTTGGCTTTTGCAGTTGGCGGTAA 359
***     *    **  **   *  *    *    ***** *     *****  * *  *

Oralis ORF2 rgg 329 AGAAGAACTATA----AACTCAATACG-ATCATTGTCAG---AGCCCTTCTGTCTTCCTG 380
Mutans ORF2 rgg 360 AGAAGCTGTTTTCTTTATTTCAGGATTCGTTGTAGCCAATCAAATCATGCAATGGACTTA 419
*****   * *     *  ***  *    *  * * **    *  * * *  *   * *

Oralis ORF2 rgg 381 TTGCTCAGATTTTCAGATTAGCAAGAAGGATATAGAATTTCTGACGGATCATCT----CT 436
Mutans ORF2 rgg 420 TGGCTTATACCTTATTGCTCAAGACAAAACTGT-GATCAACTGGCGCTCTATTTTAGTCA 478
* *** * *  **     *    * **   * * **    *** **    ** *    *

Oralis ORF2 rgg 437 TTTCTGTTGAGGAGTGGGGACGTTATGAACTCTGGCTCTTTACAAACAGCGTTGATTTGA 496
Mutans ORF2 rgg 479 ATCCTGCT-ATGATAGCAACAGTCATTGGC-CTCTTTCTTTTTATCCAACCTTT-TAAAC 535
* *** * * **  *     ** **   * **   *****  *  ** * **  *

Oralis ORF2 rgg 497 TGACCTTGGAAACGTTGGAAATCTTCGCTAGTGA-GATGATCAATCGTACCCAGTTTTAT 555
Mutans ORF2 rgg 536 TGCCTTTAGTAGCAAGAGATGCTATTGATGCTTTTGCTGATTTAA-ATACGCCTCTATCC 594
** * ** * * *    **     * * *  *   * ****  *   *** *   * *

Oralis ORF2 rgg 556 AACAACCTACCGGAAAATCGCCGCCGTATTATCAAGATGCTACTTAATGTCATTAGC--G 613
Mutans ORF2 rgg 595 ACCATTGTCTTGGGTTCTTATTTCTATAAAGTAAAATTTAAAGAAGTTTTTCTTTACTGG 654
* **   *   **    *     *  **   * **  *   *     * *  **  *  *

Oralis ORF2 rgg 614 TCTGTATAGAAGGAAACCATCT---GCTAGTT-GCTATGAGGTTTCTCAATTATCT--CG 667
Mutans ORF2 rgg 655 CCCGCATATTATACTGCCTTTTTACGCTTGTTTGTTACGGCCTTAATCAGTATTTTAACG 714
* * ***  *     ** * *   *** *** * ** *   **  *** *  * *  **

Oralis ORF2 rgg 668 ATCACTCTAAAATCC-CTGAAACAGATC-TATATGATCGAACGCTGATTAAGTATCATAG 725
Mutans ORF2 rgg 715 ATCTGGTTATTGCCCATTCATTCGGATCCTGTAAAATT-AGCCCTG-TCAATTGCTGTTA 772
***    **    **  * *  * **** * **  **  * * *** * ** *    *

Oralis ORF2 rgg 726 GGCTCTGTATGCCTACAAGGTCGGGAATACT-AATGCTCTCAGTGACATCGAGCAATGCC 784
Mutans ORF2 rgg 773 TTTCACCTGCTGCTTTAAACACGGCTCTCCTCAGTCAGGTTTATGGCGGAGAATA-TGAA 831
*    **  **   ***   * ** * *    *   ** *   **  * **

Oralis ORF2 rgg 785 TATCTTTT---TTTGAATTTTTAGATTCCTTTGGTGTTGCCCAAAAGC------TTAAAG 835
Mutans ORF2 rgg 832 TATGGTTCGCGTCTGGTCTTGTTGACAACTGTTTTGTCTCTCCTGACCATTCCTTTAAAT 891
***  **    * **   ** * **   ** *  ***  * *   * *      *****

Oralis ORF2 rgg 836 A--ACAGTTTGAAAGAATTTGCCTTTCATAG 864
Mutans ORF2 rgg 892 ATGACTGTTTCTAGCTTATTGTATTT-ATAA 921
```

10.1.8 *S. mutans gpsB* gene sequence aligned against *S. oralis gpsB* gene sequence.

```
Mutans UA159 Gp   1 ATGGCAAGTATTATGTACACCCCTAAAGATATTTTTGAACAAGAATTTAAATCTAGCATG  60
Oralis Uo5 GpsB   1 ATGGCAAGTATTATTTTTTCAGCGAAAGATATTTTTGAACAAGAATTTGGACGTGAAGTA  60
                    ************** *   *  * ************************  *  *    *

Mutans UA159 Gp  61 CGTGGCTATGATAAAAAAGAAGTTGACGAATTCCTTGACGATATTATTAAGGATTATGAA 120
Oralis Uo5 GpsB  61 CGTGGATACAGCAAAGCAGAGGTAGATGAATTCCTAGATGATGTGATTAAGGACTATGAA 120
                    ***** **    ***  *** ** ** ******** ** *** * ******** ******

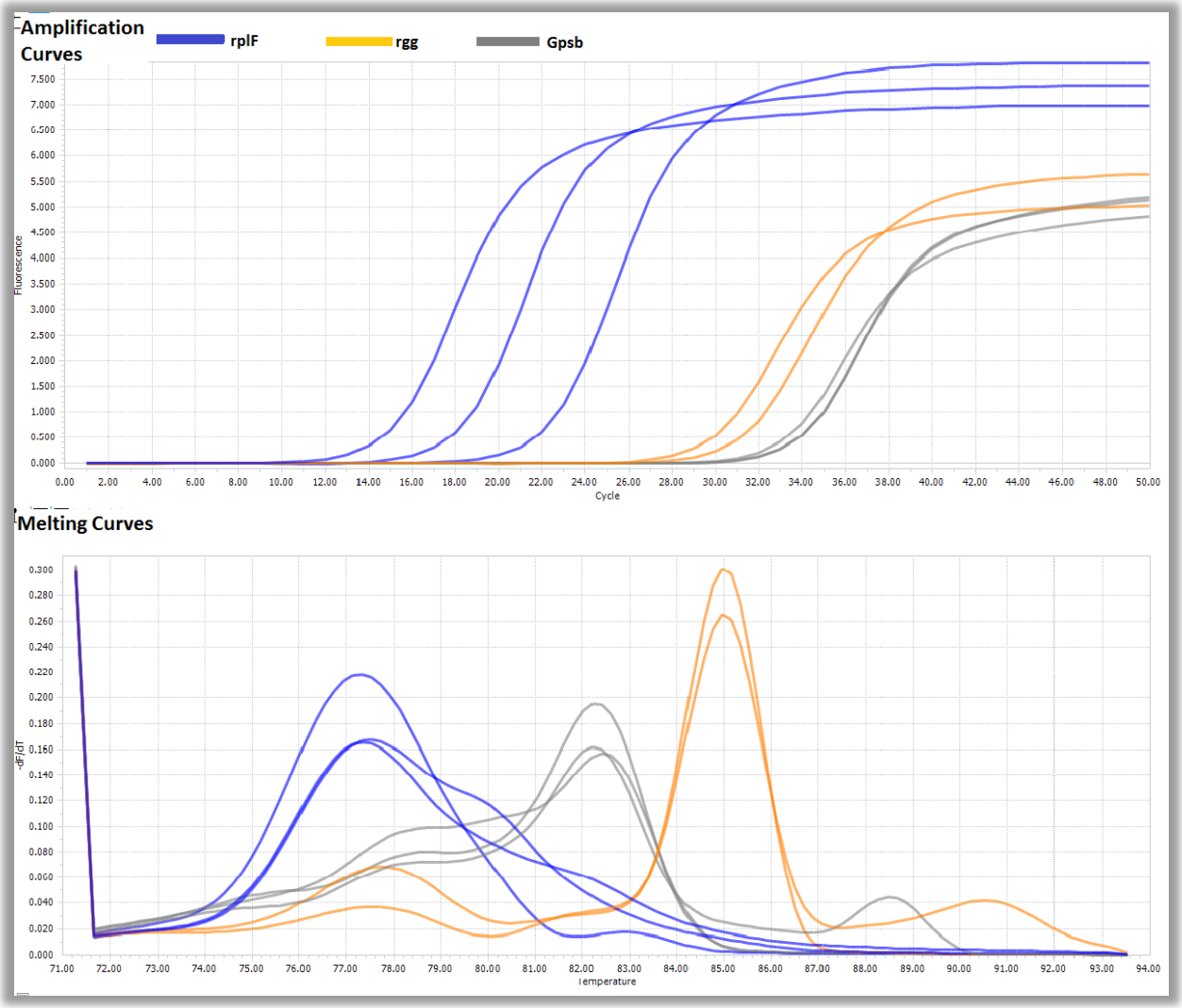
Mutans UA159 Gp 121 ACCTATATTTCCACAATCGAAGAATTGCGTCAAGAAAATACGCGCTTAAAAGAAGAAGTA 180
Oralis Uo5 GpsB 121 ACCTACGCAGCTTTGGTCAAATCCCTTCGTCAAGAGATTGCTGATTTGAAGGAAGAATTA 180
                    *****     *     ** **    * ******** * * *    ** ** ****** **

Mutans UA159 Gp 181 AAACAAGCTAAAAAAACGTCAAGAGGCCGCTCAAACAACAGTATCTCCAGCAGCTTCTGTA 240
Oralis Uo5 GpsB 181 T------CTCATAAAC------------CACAGGTAGCGCCAACTCAACCAGACTCTATT 222
                           ** * ****            * **   * *   * *** * ***  *** *

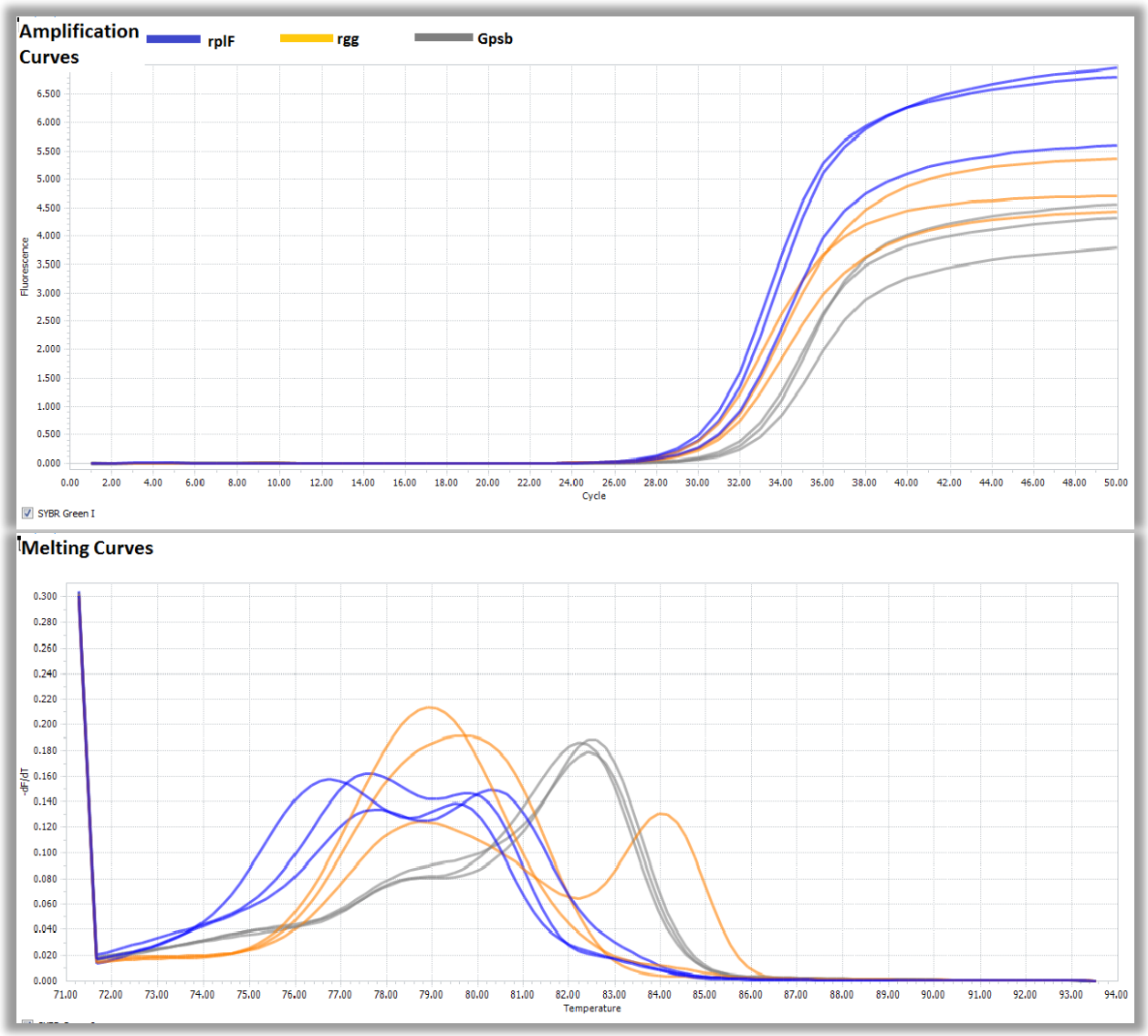
Mutans UA159 Gp 241 AGTTCTAGTCGTGTGGCGACTACCGCTACAAATTTTGATATTTTAAAACGTATCAGTCGT 300
Oralis Uo5 GpsB 223 GAAG-TAACAGCTT--CTACTTCAATGACAAACTTTGATATTTTGAAACGCTTAAATCGT 279
                         **   *  *  * *** *    ***** ********** *****  * * ****

Mutans UA159 Gp 301 TTAGAAAAAGAAGTTTTTGGTAAACAAATTACCGAATAG             339
Oralis Uo5 GpsB 280 CTCGAAAAAGAAGTATTTGGTAAGCAAATCTTAGACAACCAAGATTTATAA 330
                     * ********** ******** *****    **  *
```

10.1.9 qPCR amplification and melting curves using *rplF*, *rgg* and *gpsB* primers on *N. subflava* DNA samples



10.1.10 qPCR amplification and melting curves using *rplF*, *rgg* and *gpsB* primers on *S. oralis* DNA samples



10.1.11 qPCR amplification and melting curves using *rplF*, *rgg* and *gpsB* primers on *S. mutans* DNA samples

Appendix 2. Experimental Raw Data

10.1.12 Experiment (6.2)

Floating				
	Total CFUs			
sample number	5mg/ml	10mg/ml	GIC	control
1	5.00E+08	2.00E+08	1.30E+08	1.10E+09
2	8.00E+08	2.50E+08	1.00E+08	1.00E+09
3	2.30E+08	2.60E+08	3.00E+08	6.00E+08
4	1.30E+08	1.20E+08	1.00E+08	8.00E+08
5	7.00E+08	4.60E+08	5.00E+08	6.10E+08
6	6.90E+08	4.40E+08	4.90E+08	5.40E+08
7	5.50E+08	6.60E+08	6.50E+08	5.20E+08
8	1.90E+08	3.80E+08	1.50E+08	1.30E+08
9	1.50E+08	1.50E+08	1.70E+08	1.40E+08
10	2.30E+08	1.30E+08	1.30E+08	1.00E+08

Floating				
	S. oralis CFUs			
sample number	5mg/ml	10mg/ml	GIC	control
1	2.90E+08	3.02E+08	6.10E+08	1.13E+09
2	2.80E+08	3.31E+08	4.50E+08	6.40E+08
3	1.70E+08	1.80E+08	2.50E+08	4.60E+08
4	3.70E+08	1.10E+08	2.20E+08	3.90E+08
5	6.80E+08	6.19E+08	4.70E+08	6.40E+08
6	4.90E+08	6.60E+08	5.40E+08	7.80E+08
7	7.00E+08	8.00E+08	6.00E+08	7.20E+08
8	2.60E+08	3.60E+08	2.80E+08	1.20E+08
9	2.40E+08	1.40E+08	3.30E+08	1.90E+08
10	2.10E+08	1.20E+08	2.10E+08	2.00E+08

Floating				
	S. mutans CFUs			
sample number	5mg/ml	10mg/ml	GIC	control
1	5.00E+07	1.70E+07	2.30E+08	3.00E+08
2	1.70E+08	7.90E+07	1.70E+08	2.20E+08
3	7.00E+07	6.00E+07	3.00E+07	5.00E+07
4	1.00E+08	1.00E+07	2.00E+07	5.00E+07
5	1.00E+08	1.10E+07	7.00E+07	3.00E+07
6	1.20E+08	1.00E+07	6.00E+07	4.00E+07
7	1.30E+08	7.00E+07	7.00E+07	8.00E+07
8	1.00E+07	2.00E+07	3.00E+07	2.00E+07
9	1.00E+07	1.00E+07	4.00E+07	1.00E+07
10	2.00E+07	1.00E+07	2.00E+07	1.00E+07

Floating				
	N. subflava CFUs			
sample number	5mg/ml	10mg/ml	GIC	control
1	4.00E+07	5.00E+07	2.00E+08	3.00E+08
2	6.00E+07	4.00E+07	0.00E+00	0.00E+00
3	2.00E+07	2.00E+07	0.00E+00	3.00E+08
4	3.00E+07	4.00E+07	0.00E+00	2.00E+08
5	8.00E+07	8.00E+07	7.00E+07	1.50E+08
6	8.00E+07	6.00E+07	7.00E+07	5.00E+07
7	6.00E+07	1.00E+08	1.00E+08	1.00E+08
8	2.00E+07	1.00E+07	2.00E+07	1.00E+07
9	2.00E+07	1.00E+07	1.00E+07	2.00E+07
10	2.00E+07	1.00E+07	1.00E+07	2.00E+07

wash	Total CFUs			
sample number	5mg/ml	10mg/ml	GIC	control
1	1.54E+07	1.30E+07	2.70E+08	1.20E+08
2	1.45E+07	1.08E+07	2.20E+08	4.40E+08
3	6.80E+07	5.50E+07	1.00E+08	1.60E+08
4	7.00E+07	6.20E+07	6.00E+07	1.80E+08
5	2.34E+07	1.98E+07	1.42E+08	2.11E+08
6	3.01E+07	2.05E+07	1.99E+08	2.01E+08
7	3.09E+07	1.89E+07	1.26E+08	2.74E+08
8	7.20E+07	8.30E+07	5.80E+07	1.60E+08
9	3.80E+07	6.60E+07	6.40E+07	1.85E+08
10	6.20E+07	8.20E+07	3.70E+07	1.10E+08

wash	*S. oralis* CFUs			
sample number	5mg/ml	10mg/ml	GIC	control
1	1.91E+08	1.11E+08	1.50E+08	3.00E+08
2	1.86E+08	2.26E+08	1.70E+08	3.70E+08
3	4.20E+08	2.00E+08	3.00E+08	3.90E+08
4	3.89E+08	1.83E+08	4.70E+08	4.20E+08
5	6.80E+08	4.50E+08	2.00E+08	3.10E+08
6	4.80E+08	4.70E+08	3.40E+08	3.50E+08
7	4.40E+08	4.20E+08	1.80E+08	4.70E+08
8	1.30E+08	1.00E+08	2.19E+08	3.40E+08
9	9.00E+07	9.00E+07	2.40E+08	3.50E+08
10	1.30E+08	2.20E+08	6.00E+07	3.40E+08

wash	*S. mutans* CFUs			
sample number	5mg/ml	10mg/ml	GIC	control
1	2.00E+06	1.00E+06	2.00E+07	2.00E+07
2	3.00E+06	3.00E+06	1.00E+07	2.00E+07
3	3.00E+07	1.00E+07	3.00E+07	1.00E+07
4	2.10E+07	7.00E+06	1.00E+07	4.00E+07
5	1.00E+07	1.00E+05	3.00E+07	1.00E+07
6	2.00E+07	1.00E+05	1.00E+07	2.00E+07
7	1.00E+07	2.00E+07	1.00E+07	2.00E+07
8	1.00E+07	1.00E+07	1.00E+06	1.00E+07
9	1.00E+07	1.00E+05	2.00E+07	2.00E+07
10	2.00E+07	2.00E+07	1.00E+05	1.00E+07

wash	*N. subflava* CFUs			
sample number	5mg/ml	10mg/ml	GIC	control
1	9.00E+06	6.25E+07	5.63E+07	5.00E+07
2	3.00E+06	6.90E+07	5.92E+07	4.95E+07
3	1.10E+07	1.13E+08	1.05E+08	9.66E+07
4	6.00E+06	1.37E+08	1.30E+08	1.23E+08
5	7.00E+06	1.12E+08	1.00E+08	8.78E+07
6	1.00E+07	1.27E+08	1.16E+08	1.05E+08
7	1.30E+07	1.27E+08	1.17E+08	1.08E+08
8	3.00E+06	6.92E+07	6.38E+07	5.83E+07
9	1.00E+06	7.97E+07	7.55E+07	7.13E+07
10	3.00E+06	6.74E+07	6.25E+07	5.76E+07

Intimately attached	Total CFUs		
sample number	5mg/ml	10mg/ml	GIC
1	5.00E+05	3.70E+05	1.50E+06
2	7.50E+05	2.90E+05	1.10E+06
3	9.10E+05	3.40E+05	1.40E+06
4	8.30E+05	4.40E+05	8.00E+05
5	3.00E+05	2.50E+05	1.07E+06
6	3.00E+05	4.00E+05	1.18E+06
7	2.00E+05	8.00E+05	1.02E+06
8	3.00E+05	2.00E+05	9.90E+05
9	2.00E+05	6.00E+05	7.90E+05
10	7.00E+05	1.00E+05	8.70E+05

Intimately attached	*S. oralis* CFUs		
sample number	5mg/ml	10mg/ml	GIC
1	2.87E+06	2.05E+06	4.30E+06
2	3.50E+06	1.31E+06	5.90E+06
3	2.26E+06	1.87E+06	4.59E+06
4	2.48E+06	1.69E+06	1.21E+06
5	1.80E+06	2.60E+06	2.90E+06
6	2.40E+06	2.10E+06	3.50E+06
7	2.80E+06	2.40E+06	4.80E+06
8	5.80E+06	9.00E+05	2.20E+06
9	3.70E+06	1.70E+06	4.90E+06
10	1.30E+06	8.50E+05	2.80E+06

Intimately attached	*S. mutans* CFUs		
sample number	5mg/ml	10mg/ml	GIC
1	2.30E+05	9.00E+05	3.00E+05
2	4.00E+05	7.00E+05	1.00E+05
3	2.40E+05	8.00E+05	6.00E+05
4	3.10E+05	7.00E+05	1.10E+05
5	3.00E+05	1.00E+05	3.00E+05
6	2.00E+05	1.00E+04	2.00E+05
7	2.00E+05	1.00E+04	1.00E+05
8	2.00E+05	1.00E+05	3.00E+05
9	1.00E+04	1.00E+04	1.00E+04
10	1.00E+05	1.00E+04	2.00E+05

Intimately attached	*N. subflava* CFUs		
sample number	5mg/ml	10mg/ml	GIC
1	5.00E+04	1.00E+04	1.20E+04
2	4.00E+04	4.00E+04	1.00E+04
3	2.00E+04	1.00E+04	1.00E+04
4	1.70E+04	2.00E+04	1.00E+03
5	1.00E+04	1.00E+04	2.00E+04
6	1.00E+04	3.00E+04	6.00E+04
7	1.00E+04	2.00E+04	1.00E+04
8	3.00E+04	1.00E+03	6.00E+04
9	1.00E+04	1.00E+04	5.00E+04
10	2.00E+04	1.00E+03	2.00E+04

10.1.13 Experiment (6.3)

Floating				
	Total CFUs			
sample	5mg/ml	10mg/ml	GIC	control
1	9.00E+06	1.04E+07	2.20E+06	2.20E+06
2	6.80E+06	7.40E+06	1.30E+06	1.80E+06
3	6.80E+06	1.32E+07	1.10E+06	1.90E+06
4	5.00E+06	7.50E+06	2.00E+06	3.20E+06
5	6.90E+06	7.90E+06	4.00E+06	7.20E+06
6	6.30E+06	6.40E+06	2.00E+06	1.80E+06
7	8.70E+06	1.02E+07	2.30E+06	2.70E+06
8	7.20E+06	6.80E+06	1.50E+06	5.50E+06
9	5.50E+06	8.50E+06	3.00E+06	2.00E+06

Floating				
	S. oralis CFUs			
sample	5mg/ml	10mg/ml	GIC	control
1	6.40E+07	1.41E+08	8.70E+07	9.60E+07
2	5.60E+07	1.57E+08	1.17E+08	7.10E+07
3	5.55E+07	1.46E+08	8.90E+07	9.10E+07
4	9.38E+07	4.66E+07	8.90E+07	3.62E+07
5	9.06E+07	5.18E+07	9.50E+07	3.19E+07
6	7.47E+07	5.81E+07	9.70E+07	3.14E+07
7	9.23E+07	1.57E+08	9.60E+07	4.40E+07
8	8.37E+07	4.68E+07	8.20E+07	8.40E+07
9	6.48E+07	4.60E+07	1.23E+08	5.30E+07

Floating				
	S. mutans CFUs			
sample	5mg/ml	10mg/ml	GIC	control
1	3.00E+06	1.00E+07	2.00E+06	4.00E+06
2	1.00E+06	7.00E+06	6.00E+06	1.00E+06
3	1.50E+06	1.00E+07	1.20E+07	3.00E+06
4	2.20E+06	3.00E+06	6.00E+06	2.20E+06
5	3.00E+06	1.00E+06	8.00E+06	5.50E+06
6	3.70E+06	3.50E+06	2.00E+06	1.00E+06
7	2.70E+06	9.00E+06	9.00E+06	2.00E+06
8	2.30E+06	4.00E+06	3.00E+06	5.00E+06
9	1.20E+06	2.00E+06	7.00E+06	1.00E+06

Floating				
		N.subflava CFUs		
sample	5mg/ml	10mg/ml	GIC	control
1	5.10E+06	9.00E+06	1.80E+06	1.00E+06
2	4.90E+06	5.50E+06	1.00E+06	1.50E+06
3	5.30E+06	1.21E+07	1.00E+06	8.00E+05
4	4.50E+06	5.10E+06	1.90E+06	2.80E+06
5	5.10E+06	5.10E+06	2.20E+06	2.10E+06
6	2.80E+06	3.30E+06	1.00E+06	1.50E+06
7	5.50E+06	9.90E+06	1.30E+06	1.60E+06
8	5.90E+06	5.20E+06	9.00E+05	3.60E+06
9	4.50E+06	6.80E+06	1.70E+06	9.00E+05

wash	Total CFUs			
sample	5mg/ml	10mg/ml	GIC	control
1	4.80E+06	7.00E+06	2.30E+06	6.20E+06
2	7.30E+06	1.40E+07	3.50E+06	4.70E+06
3	6.30E+06	9.00E+06	2.70E+06	3.20E+06
4	1.40E+06	1.08E+07	3.00E+06	1.10E+06
5	3.00E+06	1.13E+07	2.00E+06	1.60E+06
6	3.20E+06	1.25E+07	2.00E+06	1.60E+06
7	2.50E+06	1.05E+07	1.80E+06	5.00E+06
8	5.60E+06	9.90E+06	2.50E+06	2.20E+06
9	1.80E+06	7.80E+06	3.00E+06	1.30E+06

wash	*S. oralis* CFUs			
sample	5mg/ml	10mg/ml	GIC	control
1	1.55E+08	9.40E+07	8.90E+07	1.07E+08
2	1.22E+08	1.26E+08	9.50E+07	1.20E+08
3	1.35E+08	1.09E+08	7.60E+07	1.01E+08
4	4.30E+07	1.45E+08	1.12E+08	6.29E+07
5	3.72E+07	1.15E+08	8.40E+07	6.13E+07
6	6.22E+07	1.27E+08	1.07E+08	4.43E+07
7	1.04E+08	1.07E+08	9.40E+07	8.39E+07
8	6.54E+07	1.46E+08	8.00E+07	8.80E+07
9	4.50E+07	1.40E+08	9.20E+07	9.55E+07

wash	*S. mutans* CFUs			
sample	5mg/ml	10mg/ml	GIC	control
1	3.00E+06	3.00E+06	1.00E+05	1.00E+05
2	1.00E+06	2.00E+06	5.00E+06	7.00E+06
3	1.00E+04	2.00E+06	2.00E+06	3.00E+06
4	1.00E+06	3.50E+06	3.00E+06	2.75E+06
5	1.00E+04	3.50E+06	2.00E+06	1.50E+06
6	1.00E+06	2.70E+06	6.00E+06	1.66E+06
7	1.50E+06	2.20E+06	8.00E+06	2.10E+06
8	1.00E+04	3.70E+06	1.00E+05	4.00E+06
9	1.00E+04	2.50E+06	1.00E+05	4.50E+06

wash *N. subflava* CFUs				
sample	5mg/ml	10mg/ml	GIC	control
1	3.00E+06	3.00E+06	1.60E+06	5.50E+06
2	4.70E+06	5.00E+06	2.60E+06	3.00E+06
3	4.50E+06	4.00E+06	2.20E+06	2.00E+06
4	1.00E+06	2.50E+06	2.00E+06	7.00E+05
5	2.20E+06	3.50E+06	1.00E+06	8.00E+05
6	2.30E+06	3.50E+06	2.00E+06	6.00E+05
7	1.70E+06	5.40E+06	1.20E+06	2.20E+06
8	3.60E+06	3.80E+06	1.80E+06	1.80E+06
9	1.50E+06	3.00E+06	2.20E+06	9.00E+05

Intimately attached	5 mg/ml			
sample	Total CFUs	*N. subflava* CFUs	*S. mutans* CFUs	*S. oralis* CFUs
1	1.60E+04	1.30E+04	2.00E+04	4.00E+05
2	1.40E+04	9.00E+03	0.00E+00	2.60E+05
3	1.20E+04	9.00E+03	0.00E+00	3.90E+05
4	1.50E+04	1.50E+04	3.00E+04	4.30E+05
5	1.30E+04	9.00E+03	2.00E+04	4.60E+05
6	1.30E+04	1.00E+04	4.00E+04	4.20E+05
7	1.50E+04	1.10E+04	2.00E+04	3.40E+05
8	1.70E+04	1.50E+04	5.00E+04	4.00E+05
9	1.20E+04	9.00E+03	0.00E+00	2.70E+05

Intimately attached	10 mg/ml			
sample	Total CFUs	*N. subflava* CFUs	*S. mutans* CFUs	*S. oralis* CFUs
1	9.10E+05	8.00E+04	3.00E+05	4.40E+06
2	7.90E+05	4.00E+04	1.00E+05	7.10E+06
3	7.50E+05	6.00E+04	2.00E+05	6.40E+06
4	5.20E+05	3.00E+04	1.00E+05	3.14E+06
5	5.80E+05	4.50E+04	1.20E+05	2.20E+06
6	5.70E+05	3.20E+04	5.00E+04	2.91E+06
7	6.90E+05	5.00E+04	2.00E+05	4.40E+06
8	8.00E+05	6.00E+04	2.00E+05	5.00E+06
9	5.50E+05	8.00E+04	1.00E+05	2.80E+06

Intimately attached	GIC			
sample	Total CFUs	*N. subflava* CFUs	*S. mutans* CFUs	*S. oralis* CFUs
1	9.30E+04	4.70E+04	8.00E+04	1.88E+06
2	5.40E+04	3.20E+04	4.00E+04	2.12E+06
3	8.00E+04	3.50E+04	6.00E+04	2.04E+06
4	5.60E+04	2.30E+04	7.00E+04	8.90E+05
5	4.70E+04	3.90E+04	4.00E+04	7.80E+05
6	5.20E+04	1.90E+04	5.00E+04	8.50E+05
7	7.70E+04	3.20E+04	5.00E+04	1.75E+06
8	5.20E+04	2.50E+04	4.00E+04	9.50E+05
9	5.50E+04	2.20E+04	7.00E+04	9.80E+05

10.1.14 Experiment (6.4)

10.1.14.1 MTT aerobic

GIC	Floating				
1. Raw Data 550 - Raw Data 620					
0.237	0.249	0.132	0.121	0.164	0.126
0.189	0.289	0.231	0.202	0.157	0.233

5mg/ml	Floating				
1. Raw Data 550 - Raw Data 620					
0.34	0.3	0.33	0.37	0.35	0.32
0.32	0.32	0.34	0.47	0.29	0.27

10mg/ml	Floating				
1. Raw Data 550 - Raw Data 620					
0.32	0.29	0.37	0.21	0.35	0.37
0.23	0.31	0.21	0.39	0.24	0.28

control	Floating				
1. Raw Data 550 - Raw Data 620					
0.41	0.62	0.71	0.44	0.53	0.61
0.72	0.72	0.56	0.42	0.66	0.61

GIC	Wash				
1. Raw Data 550 - Raw Data 620					
0.237	0.249	0.132	0.121	0.164	0.126
0.189	0.289	0.231	0.202	0.157	0.233

5mg/ml	Wash				
1. Raw Data 550 - Raw Data 620					
0.163	0.129	0.185	0.145	0.181	0.109
0.148	0.154	0.186	0.114	0.141	0.093

10mg/ml	Wash				
1. Raw Data 550 - Raw Data 620					
0.131	0.147	0.141	0.132	0.107	0.102
0.187	0.153	0.14	0.101	0.14	0.122

control	Wash				
1. Raw Data 550 - Raw Data 620					
0.26	0.15	0.27	0.19	0.26	0.28
0.14	0.22	0.28	0.18	0.23	0.27

GIC	Intimately attached				
1. Raw Data 550 - Raw Data 620					
0.237	0.249	0.132	0.121	0.164	0.126
0.189	0.289	0.231	0.202	0.157	0.233

5mg/ml	Intimately attached				
1. Raw Data 550 - Raw Data 620					
0.014	0.02	0.003	0.018	0.007	0.007
0.003	0.018	0.003	0.011	0.017	0.008

10mg/ml	Intimately attached				
1. Raw Data 550 - Raw Data 620					
0.004	0.005	0.009	0.02	0.02	0.005
0.029	0.015	0.022	0	0.013	0.004

10.1.14.2 MTT anaerobic

GIC	Floating				
1. Raw Data 550 - Raw Data 620					
0.237	0.249	0.132	0.121	0.164	0.126
0.189	0.289	0.231	0.202	0.157	0.233

5mg/ml	Floating				
1. Raw Data 550 - Raw Data 620					
0.11	0.15	0.11	0.12	0.12	0.12
0.13	0.15	0.07	0.11	0.11	0.09

10mg/ml	Floating				
1. Raw Data 550 - Raw Data 620					
0.1	0.11	0.15	0.16	0.14	0.11
0.17	0.17	0.32	0.11	0.13	0.11

control	Floating				
1. Raw Data 550 - Raw Data 620					
0.14	0.11	0.1	0.06	0.05	0.12
0.08	0.12	0.04	0.11	0.12	0.07

GIC	Wash				
1. Raw Data 550 - Raw Data 620					
0.237	0.249	0.132	0.121	0.164	0.126
0.189	0.289	0.231	0.202	0.157	0.233

5mg/ml	Wash				
1. Raw Data 550 - Raw Data 620					
0.036	0.018	0.025	0.02	0.011	0.025
0.042	0.034	0.016	0.034	0.021	0.03

10mg/ml	Wash				
1. Raw Data 550 - Raw Data 620					
0.097	0.074	0.046	0.05	0.09	0.052
0.051	0.071	0.072	0.093	0.055	0.072

control	Wash				
1. Raw Data 550 - Raw Data 620					
0.025	0.018	0.031	0.04	0.045	0.039
0.035	0.042	0.034	0.035	0.041	0.034

GIC	Intimately attached				
1. Raw Data 550 - Raw Data 620					
0.237	0.249	0.132	0.121	0.164	0.126
0.189	0.289	0.231	0.202	0.157	0.233

5mg/ml	Intimately attached				
1. Raw Data 550 - Raw Data 620					
0.001	0.005	0	0.001	0.003	0
0	0.003	0.002	0	0	0.001

10mg/ml	Intimately attached				
1. Raw Data 550 - Raw Data 620					
0.005	0.005	0.008	0.007	0.004	0.006
0.01	0.003	0.006	0.006	0.004	0.005

10.1.15 Experiment (6.5)

Boiling	5mg/ml Ag+ Aerobic	5mg/ml Ag+ Anaerobic	10mg/ml Ag+ Aerobic	10mg/ml Ag+ Anaerobic	GIC Aerobic	GIC Anaerobic
1	47.5	51	27.5	52	38.5	48.5
2	47	47	52	68.5	37	22.3
3	31.5	57.2	4.2	174	33	23
4	40.3	61.5	4.8	59.5	42	35.3
5	53	55.4	5.5	91	47	45
6	42.5	44.2	4.6	72	22	68.5
7	41.6	50	2.7	102	35	51
8	32	35	4.8	56	40	37
9	37.2	66	5.8	82	32.5	22

DNeasy kit	5mg/ml Ag+ Aerobic	5mg/ml Ag+ Anaerobic	10mg/ml Ag+ Aerobic	10mg/ml Ag+ Anaerobic	GIC Aerobic	GIC Anaerobic
1	7	2	7.6	2.7	3.7	1.7
2	4	2.7	4.1	2.2	3.4	2.2
3	4.2	2.7	4.2	2	2.9	1.9
4	4	1.9	4.8	3.5	4.9	2.1
5	5.8	2.2	5.5	5	4.2	3.2
6	5.4	3.2	4.6	2.1	2.3	2
7	4	2.6	2.7	4.7	6.8	2.3
8	3.8	2.8	4.8	3.8	3.2	3.5
9	5.6	2.8	5.8	3.4	2	4

10.1.16 Experiment (6.6)

	Floating			Wash			Intimately attached			
Sample	5mg/ml	10mg/ml	GIC	control	5mg/ml	10mg/ml	GIC	control	5mg/ml	10mg/ml	GIC
1	3.00E+03	3.00E+06	9.00E+04	1.70E+06	1.00E+05	1.00E+06	1.20E+05	5.30E+06	5.00E+03	2.53E+06	1.20E+05
2	2.00E+03	2.70E+06	1.10E+05	1.60E+06	2.00E+04	2.00E+06	2.00E+05	4.00E+06	2.00E+03	1.98E+06	2.00E+05
3	7.00E+03	3.30E+06	1.50E+05	1.90E+06	4.00E+04	2.00E+06	1.50E+05	3.20E+06	3.00E+03	1.36E+06	1.50E+05
4	2.00E+03	1.60E+06	1.20E+05	2.20E+06	5.00E+04	1.00E+06	1.70E+05	2.70E+06	2.00E+03	1.23E+06	1.70E+05
5	1.00E+03	3.10E+06	1.60E+05	1.10E+06	2.00E+04	3.00E+06	1.60E+05	5.60E+06	1.00E+03	1.51E+06	1.60E+05
6	2.00E+03	2.90E+06	2.20E+05	1.60E+06	8.00E+04	7.00E+06	2.40E+05	4.30E+06	1.00E+03	2.36E+06	2.40E+05

10.1.17 Experiment (6.7)

floating			
Total CFUs			
sample	GIC	10mg/ml	PVA
1	5.00E+06	1.40E+07	2.20E+07
2	2.20E+06	1.70E+07	2.80E+07
3	4.10E+06	8.90E+06	9.00E+06
4	3.90E+06	9.50E+06	1.20E+07
5	4.00E+06	9.90E+06	7.90E+06
6	2.50E+06	1.20E+07	8.80E+06

floating			
S. oralis CFUs			
sample	GIC	10mg/ml	PVA
1	5.00E+07	8.60E+07	6.59E+07
2	7.01E+07	4.60E+07	9.50E+07
3	6.20E+07	9.00E+07	9.60E+07
4	8.89E+07	1.35E+08	1.20E+08
5	9.00E+07	9.00E+07	1.89E+08
6	8.99E+07	7.50E+07	1.28E+08

floating			
S. mutans CFUs			
sample	GIC	10mg/ml	PVA
1	1.00E+06	1.00E+07	1.00E+05
2	9.00E+05	1.00E+06	1.00E+06
3	2.00E+06	1.50E+07	3.00E+06
4	1.00E+05	3.00E+07	1.00E+05
5	2.00E+06	1.00E+06	7.00E+06
6	1.00E+05	8.00E+06	5.00E+06

floating			
N. subflava CFUs			
sample	GIC	10mg/ml	PVA
1	2.00E+06	8.00E+06	7.00E+06
2	1.20E+06	9.00E+06	9.00E+06
3	1.80E+06	3.60E+06	4.00E+06
4	1.40E+06	4.60E+06	1.00E+06
5	2.30E+06	3.80E+06	3.40E+06
6	9.00E+05	6.10E+06	4.90E+06

Wash			
Total CFUs			
sample	GIC	10mg/ml	PVA
1	1.08E+07	9.00E+06	1.80E+07
2	8.60E+06	7.00E+06	2.00E+07
3	8.40E+06	1.20E+07	1.30E+07
4	7.90E+06	8.60E+06	1.20E+07
5	8.70E+06	1.08E+07	1.70E+07
6	9.60E+06	9.50E+06	9.00E+06

Wash			
S. oralis CFUs			
sample	GIC	10mg/ml	PVA
1	1.40E+08	2.00E+08	2.00E+08
2	9.00E+07	1.70E+08	1.50E+08
3	8.60E+07	1.10E+08	1.97E+08
4	8.39E+07	1.49E+08	1.20E+08
5	8.00E+07	2.60E+08	1.12E+08
6	9.70E+07	1.19E+08	1.80E+08

Wash			
S. mutans CFUs			
sample	GIC	10mg/ml	PVA
1	1.00E+07	2.00E+07	2.00E+07
2	4.00E+06	1.00E+07	1.00E+07
3	2.00E+07	2.00E+07	3.00E+06
4	1.00E+05	1.00E+06	1.00E+05
5	2.00E+06	1.00E+07	4.00E+06
6	2.00E+06	1.00E+06	1.00E+05

Wash			
N. subflava CFUs			
sample	GIC	10mg/ml	PVA
1	4.00E+06	3.00E+06	5.00E+06
2	1.80E+06	1.00E+06	1.20E+07
3	2.80E+06	4.00E+06	7.00E+06
4	3.60E+06	2.20E+06	5.00E+06
5	2.30E+06	4.50E+06	3.00E+06
6	1.20E+06	6.10E+06	4.00E+06

Intimately attached			
Total CFUs			
sample	GIC	10mg/ml	PVA
1	1.60E+04	1.26E+06	9.50E+05
2	3.40E+04	1.06E+06	1.04E+06
3	1.30E+04	9.50E+05	6.30E+05
4	4.50E+04	9.90E+05	7.50E+05
5	2.70E+04	1.02E+06	8.30E+05
6	1.90E+04	8.70E+05	9.00E+05

Intimately attached			
S. oralis CFUs			
sample	GIC	10mg/ml	PVA
1	7.70E+05	4.40E+06	4.70E+06
2	4.10E+05	5.40E+06	3.59E+06
3	2.00E+05	3.49E+06	2.80E+06
4	4.90E+05	6.40E+06	5.49E+06
5	5.90E+05	2.10E+06	2.49E+06
6	2.50E+05	3.29E+06	4.40E+06

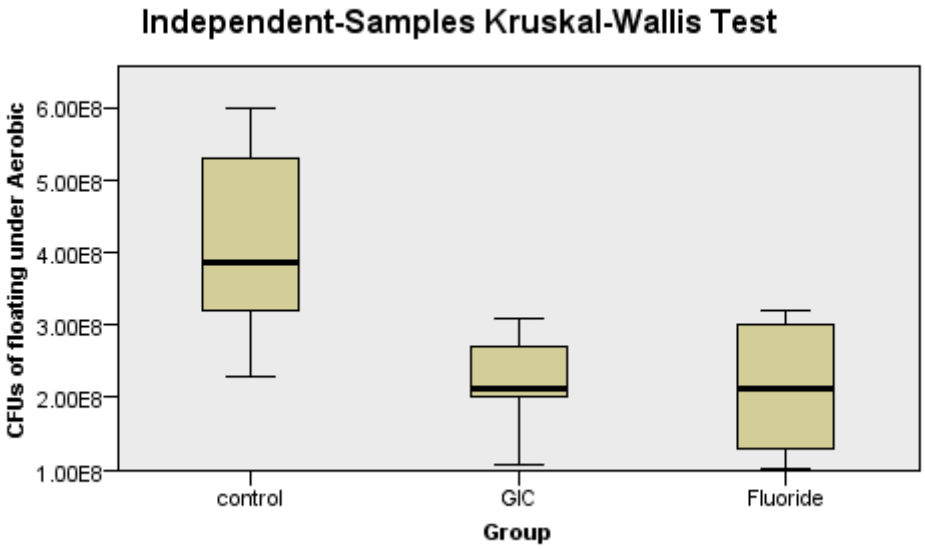
Intimately attached			
S. mutans CFUs			
sample	GIC	10mg/ml	PVA
1	3.00E+04	1.00E+05	2.00E+05
2	4.00E+04	1.00E+05	1.00E+04
3	9.00E+04	1.00E+04	1.00E+05
4	7.00E+04	1.00E+05	1.00E+04
5	7.00E+04	3.00E+05	1.00E+04
6	5.00E+04	1.00E+04	2.00E+05

Intimately attached			
N. subflava CFUs			
sample	GIC	10mg/ml	PVA
1	6.00E+03	3.50E+05	2.00E+04
2	9.00E+03	2.90E+05	2.90E+04
3	7.00E+03	1.80E+05	1.80E+04
4	3.00E+03	1.00E+05	1.00E+04
5	4.00E+03	3.30E+05	9.00E+03
6	1.00E+03	3.70E+05	1.30E+04

10.2 Appendix 3. Statistical Analysis Results

10.2.1 Experiment (5.8)

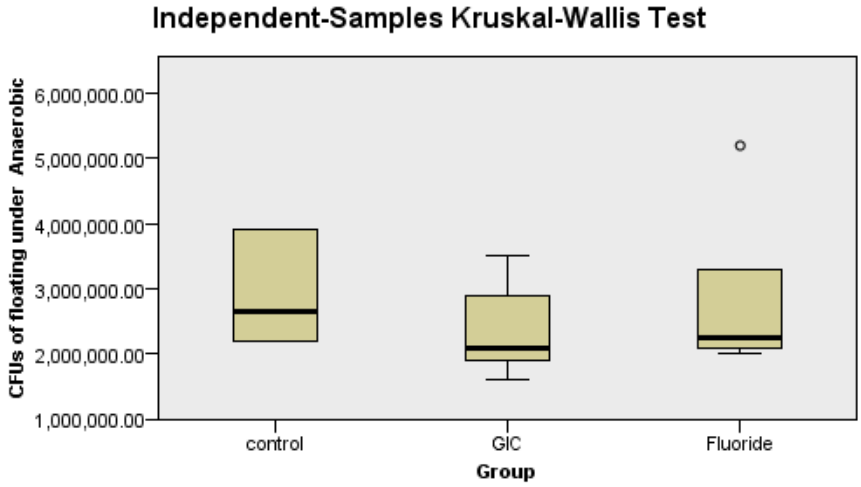
10.2.1.1 Floating assay aerobic.



Total N	18
Test Statistic	8.175
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.017

1. The test statistic is adjusted for ties.

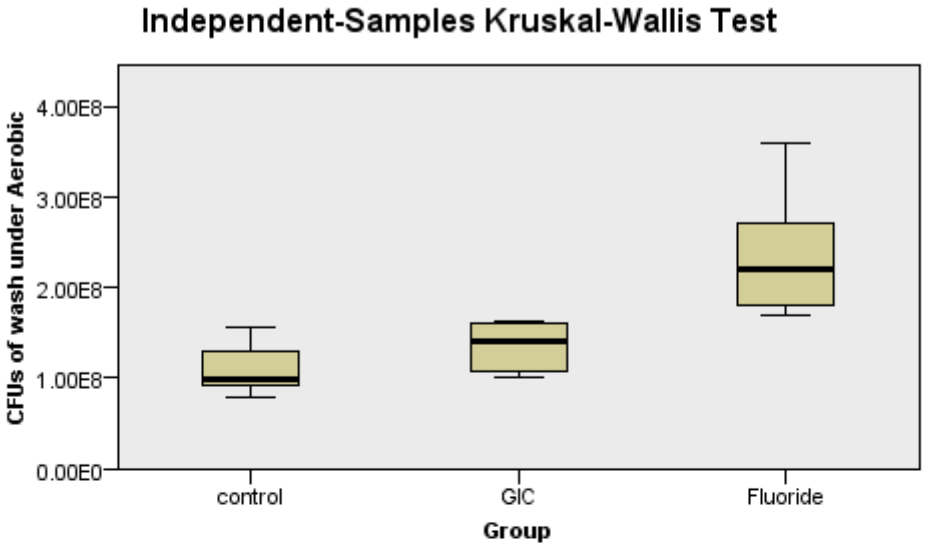
10.2.1.2 Floating assay anaerobic.



Total N	18
Test Statistic	2.499
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.287

1. The test statistic is adjusted for ties.
2. Multiple comparisons are not performed because the overall test does not show significant differences across samples.

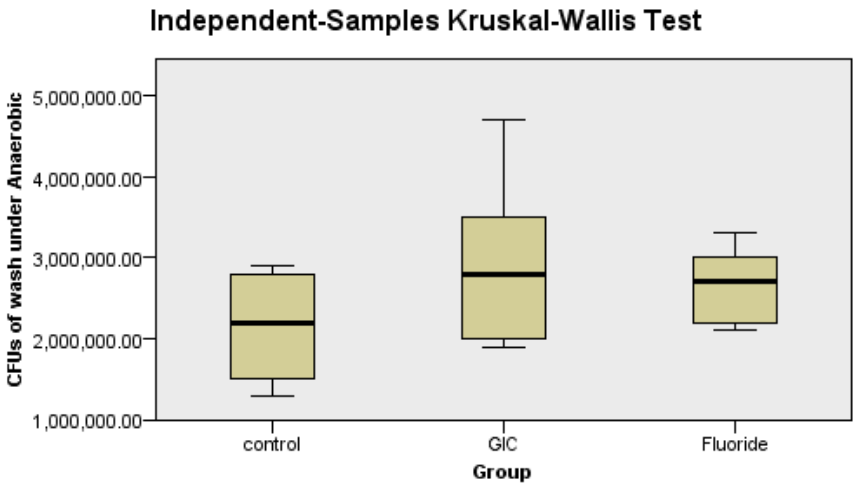
10.2.1.3 Wash assay aerobic.



Total N	18
Test Statistic	12.929
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.002

1. The test statistic is adjusted for ties.

10.2.1.4 Wash assay anaerobic.



Total N	18
Test Statistic	2.271
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.321

1. The test statistic is adjusted for ties.
2. Multiple comparisons are not performed because the overall test does not show significant differences across samples.

10.2.2 Experiment (6.2)

10.2.2.1 Floating assay aerobic after 24Hrs.

A

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of mg_5, mg_10, GIC and Control are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.513	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

B

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of mg5, mg10, GIC and Control are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.230	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

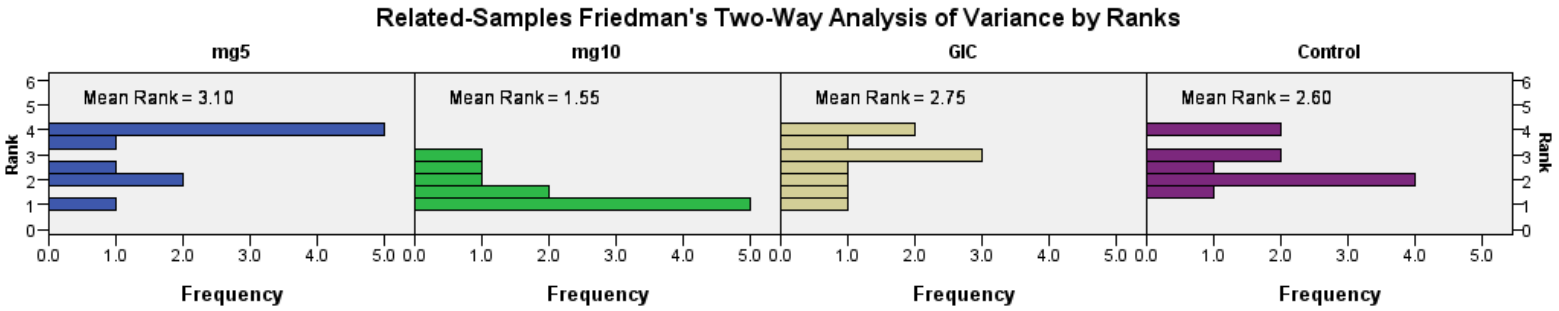
C

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of mg5, mg10, GIC and Control are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.323	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

(A)Total. (B) *S. oralis*. (C) *N. subflava* CFUs related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. (*p>0.05*). Sig. = Significance



Total N	10
Test Statistic	8.802
Degrees of Freedom	3
Asymptotic Sig. (2-sided test)	.032

S. mutans CFUs related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance level

10.2.2.2 Wash assay aerobic after 24Hrs.

Total N	10
Test Statistic	17.160
Degrees of Freedom	3
Asymptotic Sig. (2-sided test)	.001

Total CFUs related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance level

A

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of mg5, mg10, GIC and Control are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.103	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

B

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of mg5, mg10, GIC and Control are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.112	Retain the null hypothesis.

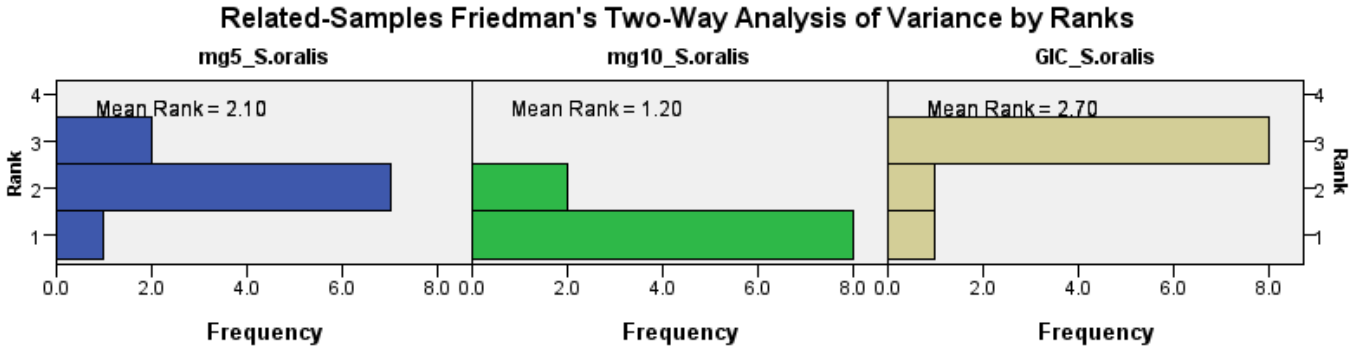
Asymptotic significances are displayed. The significance level is .05.

(A) *S. mutans*. (B) *N. subflava* CFUs related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance.

10.2.2.3 Intimately attached assay aerobic after 24Hrs.

Total N	10
Test Statistic	13.400
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.001

Total CFUs related Friedman's two-way ANOVA by rank test results showing significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance level



Total N	10
Test Statistic	11.400
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.003

S. oralis CFUs related Friedman's two-way ANOVA by rank test results showing significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance level

A

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of mg5_N.subflava, mg10_N.subflava and GIC_N. subflava are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.682	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

B

Hypothesis Test Summary

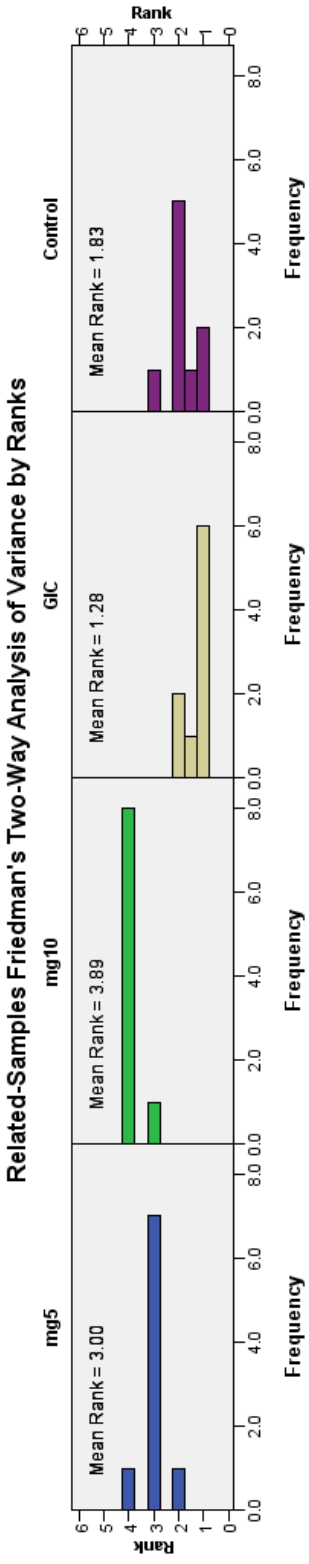
	Null Hypothesis	Test	Sig.	Decision
1	The distributions of mg5_S.mutans, mg10_S.mutans and GIC_S. mutans are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.889	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

(A) *N. subflava.* (B) *S. mutans* CFUs related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance.

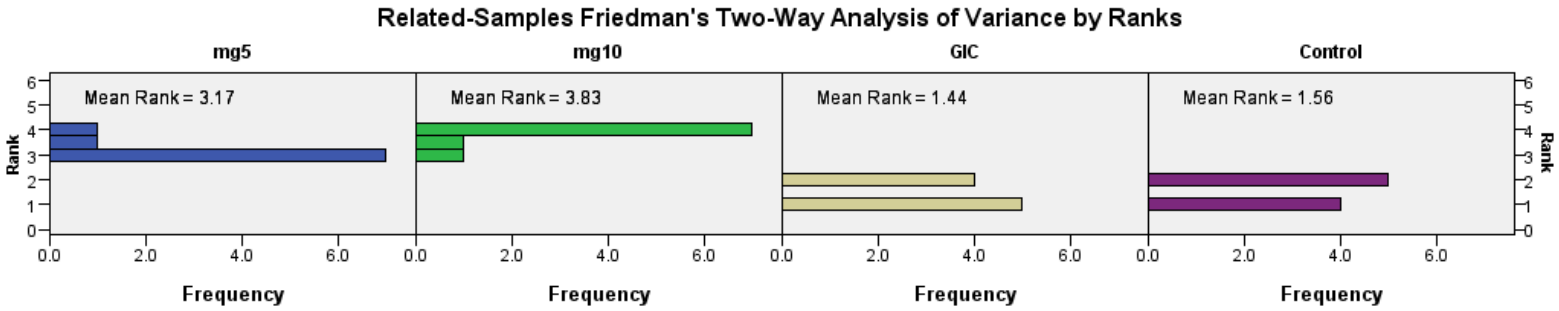
10.2.3 Experiment (6.3)

10.2.3.1 Floating assay anaerobic after 24Hrs.



Total N	9
Test Statistic	22.483
Degrees of Freedom	3
Asymptotic Sig. (2-sided test)	.000

Total floating CFUs related Friedman's two-way ANOVA by rank test results showing significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p > 0.05$). Sig. = Significance level



Total N	9
Test Statistic	23.090
Degrees of Freedom	3
Asymptotic Sig. (2-sided test)	.000

N. subflava floating CFUs related Friedman's two-way ANOVA by rank test results showing significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. (*p>0.05*). Sig. = Significance level

A

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of mg5, mg10, GIC and Control are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.074	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

B

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of mg5, mg10, GIC and Control are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.392	Retain the null hypothesis.

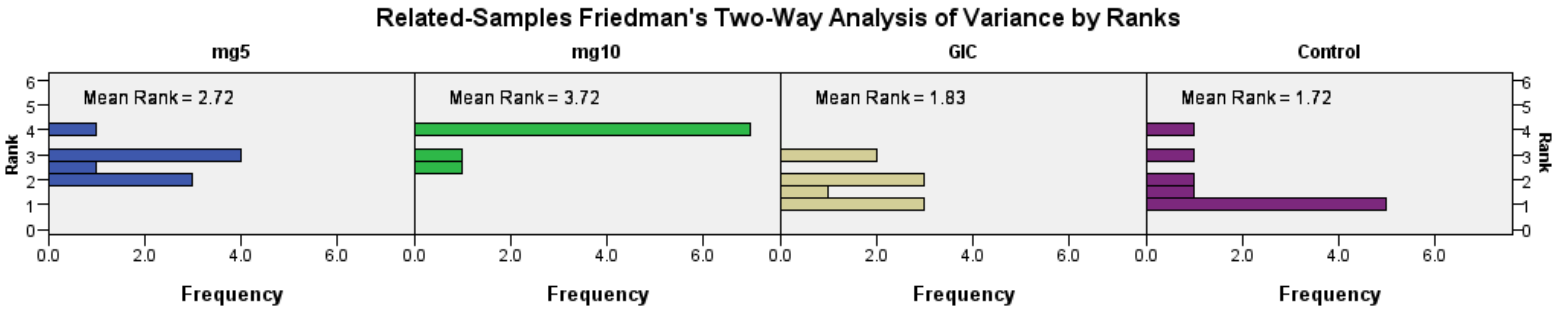
Asymptotic significances are displayed. The significance level is .05.

(A) *S. oralis.* (B) *S. mutans* CFUs related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance.

10.2.3.2 Wash assay anaerobic after 24Hrs.

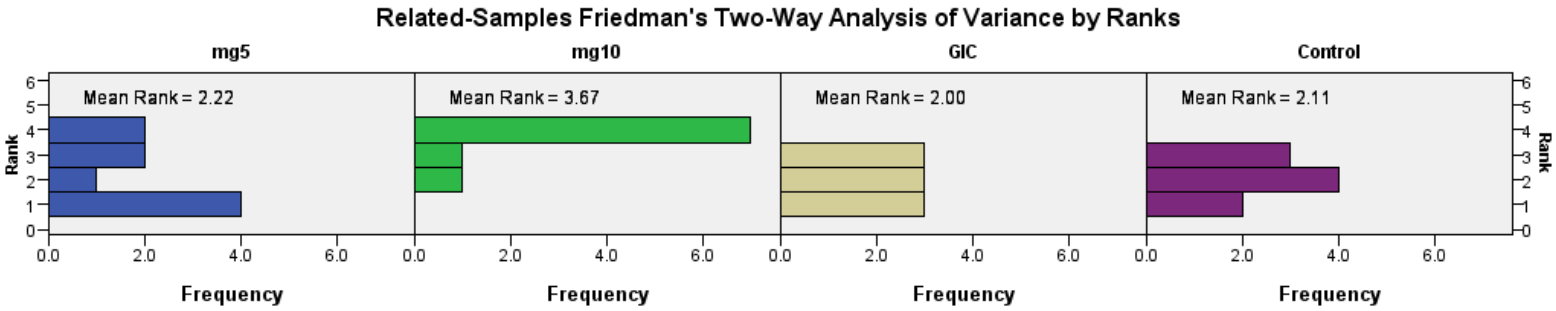
Total N	9
Test Statistic	18.733
Degrees of Freedom	3
Asymptotic Sig. (2-sided test)	.000

Total CFUs related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance level



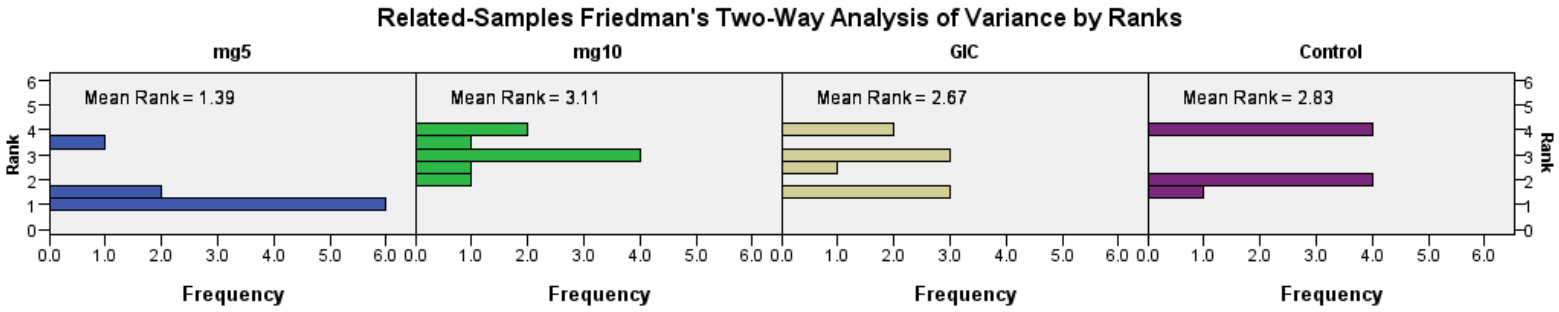
Total N	9
Test Statistic	14.318
Degrees of Freedom	3
Asymptotic Sig. (2-sided test)	.003

N. subflava related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. (*p>0.05*). Sig. = Significance level



Total N	9
Test Statistic	9.933
Degrees of Freedom	3
Asymptotic Sig. (2-sided test)	.019

S. oralis related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. (*p>0.05*). Sig. = Significance level



Total N	9
Test Statistic	9.988
Degrees of Freedom	3
Asymptotic Sig. (2-sided test)	.019

S. mutans related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance level

10.2.4 Experiment (6.4)

10.2.4.1 MTT Floating assay aerobic

Total N	12
Test Statistic	24.731
Degrees of Freedom	3
Asymptotic Sig. (2-sided test)	.000

MTT related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance level

10.2.4.2 MTT Wash assay aerobic

Total N	12
Test Statistic	20.500
Degrees of Freedom	3
Asymptotic Sig. (2-sided test)	.000

MTT related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance level

10.2.4.3 MTT Intimately attaches assay aerobic.

Total N	12
Test Statistic	15.167
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.001

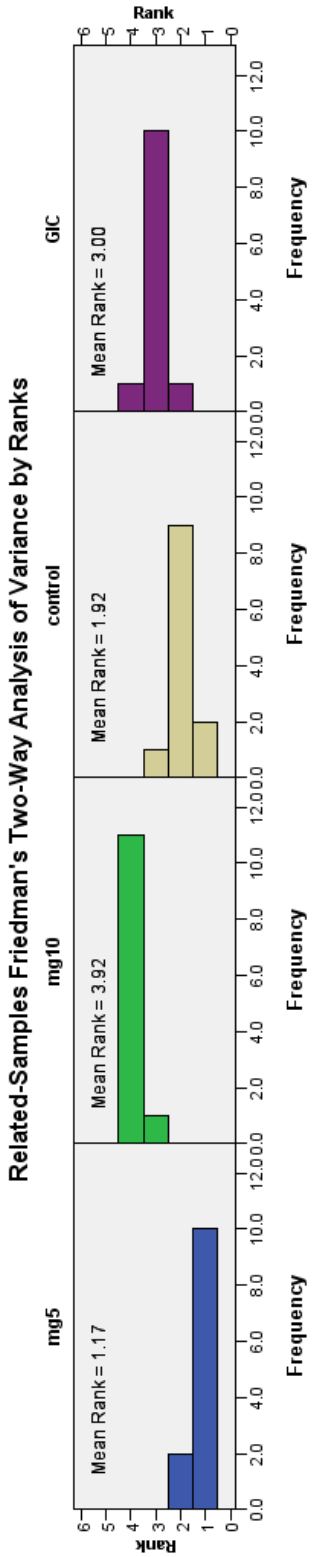
MTT related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance level

10.2.4.4 MTT Floating assay anaerobic

Total N	12
Test Statistic	18.802
Degrees of Freedom	3
Asymptotic Sig. (2-sided test)	.000

MTT related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance level

10.2.4.5 MTT Wash assay anaerobic



Total N	12
Test Statistic	31.500
Degrees of Freedom	3
Asymptotic Sig. (2-sided test)	.000

MTT related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance level

10.2.4.6 MTT Intimately attached assay anaerobic.

Total N	12
Test Statistic	16.870
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.000

MTT related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance level

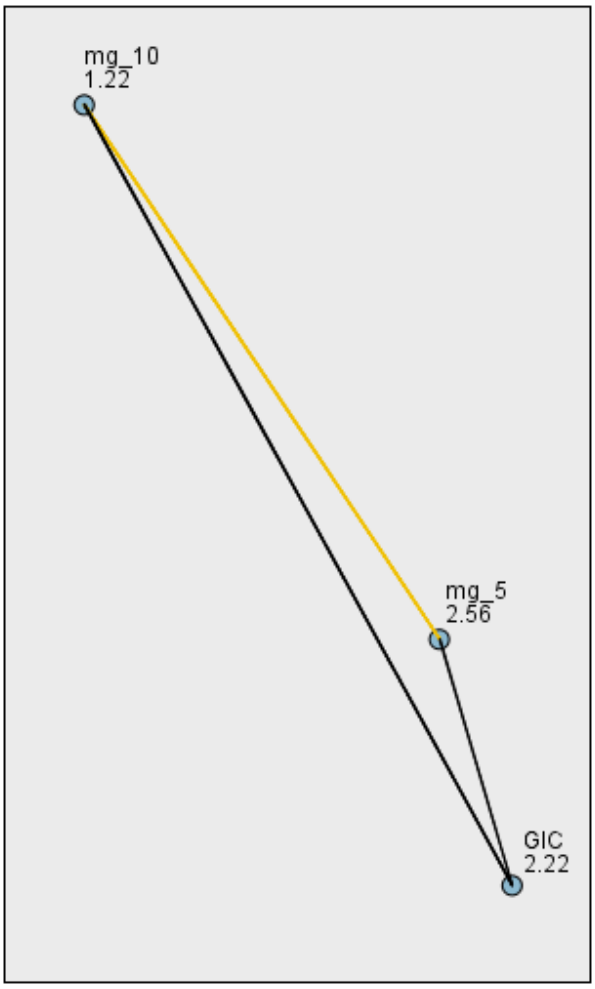
10.2.5 Experiment (6.5)

10.2.5.1 Boiling aerobic

Total N	9
Test Statistic	8.667
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.013

DNA quantities from Boiling method related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance level

Pairwise Comparisons



Each node shows the sample average rank.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
mg_10-GIC	-1.000	.471	-2.121	.034	.102
mg_10-mg_5	1.333	.471	2.828	.005	.014
GIC-mg_5	.333	.471	.707	.480	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

Pairwise comparison of related DNA quantities from Boiling method samples showing significant difference DNA samples from 10mg/ml silver modified and 5mg/ml silver modified GIC disks. ($p>0.05$). Sig. = Significance level

10.2.5.2 Boiling anaerobic

Total N	9
Test Statistic	11.556
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.003

DNA quantities from Boiling method related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p > 0.05$). Sig. = Significance level

Pairwise Comparisons

Each node shows the sample average rank.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
GIC-mg_5	.444	.471	.943	.346	1.000
GIC-mg_10	1.556	.471	3.300	.001	.003
mg_5-mg_10	-1.111	.471	-2.357	.018	.055

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

Pairwise comparison of related DNA quantities from Boiling method samples showing significant difference DNA samples from 10mg/ml silver modified and 5mg/ml silver modified GIC disks. ($p>0.05$). Sig. = Significance level.

10.2.5.3 DNeasy Kit aerobic

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of mg_5, mg_10 and GIC are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.105	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

DNA quantities from DNeasy kit method related Friedman's two-way ANOVA by rank test

10.2.5.4 DNeasy kit Anaerobic.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of mg_5, mg_10 and GIC are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.147	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

DNA quantities from DNeasy kit method related Friedman's two-way ANOVA by rank test

10.2.6 Experiment (6.6)

10.2.6.1 Floating assay anaerobic 48Hrs.

Total N	6
Test Statistic	17.000
Degrees of Freedom	3
Asymptotic Sig. (2-sided test)	.001

S. oralis related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance level

10.2.6.2 Wash assay anaerobic 48Hrs.

Total N	6
Test Statistic	17.000
Degrees of Freedom	3
Asymptotic Sig. (2-sided test)	.001

S. oralis related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance level

10.2.6.3 Intimately attached assay anaerobic 48Hrs.

Total N	6
Test Statistic	12.000
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.002

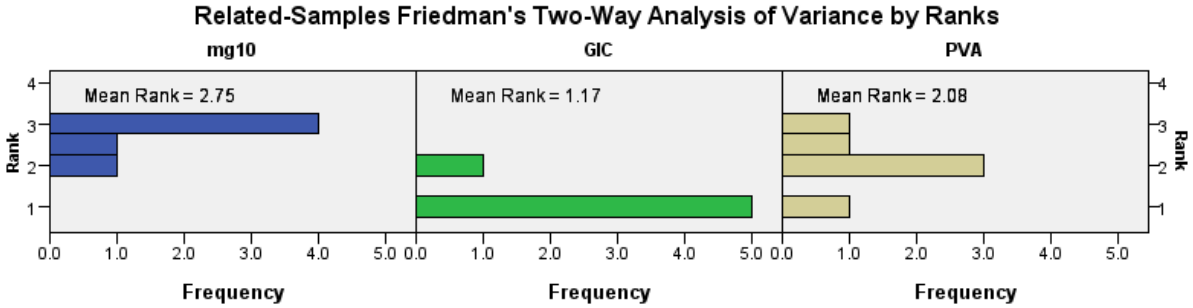
S. oralis related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance level

10.2.7 Experiment (6.7)

10.2.7.1 Floating assay anaerobic 24Hrs.

Total N	6
Test Statistic	9.333
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.009

Total CFUs related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p > 0.05$). Sig. = Significance level



Total N	6
Test Statistic	7.913
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.019

N. subflava related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance level

A

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of mg10, GIC and PVA are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.084	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

B

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of mg10, GIC and PVA are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.084	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

(A) *S. oralis.* (B) *S. mutans* CFUs related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance.

10.2.7.2 Wash assay anaerobic 24Hrs.

A

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of mg10, GIC and PVA are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.135	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

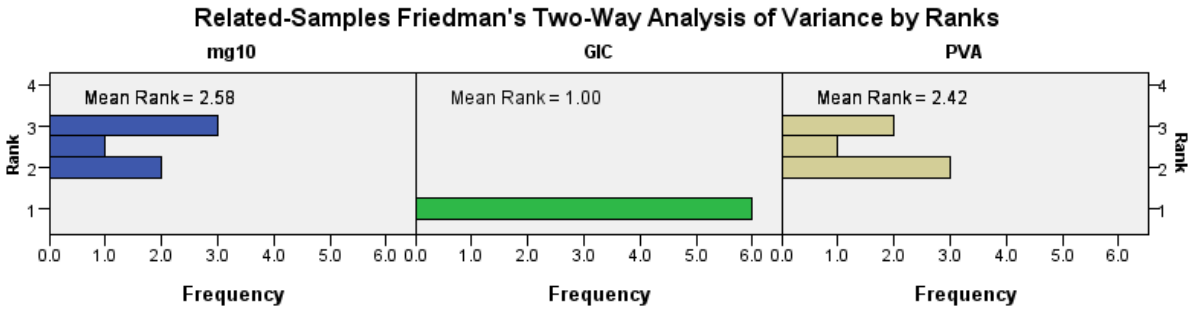
B

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of mg10, GIC and PVA are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.115	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

(A) Total (B) *N. subflava* CFUs related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance.



Total N	6
Test Statistic	9.478
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.009

Total CFUs related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. (*p>0.05*). Sig. = Significance level

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of mg10, GIC and PVA are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.157	Retain the null hypothesis.

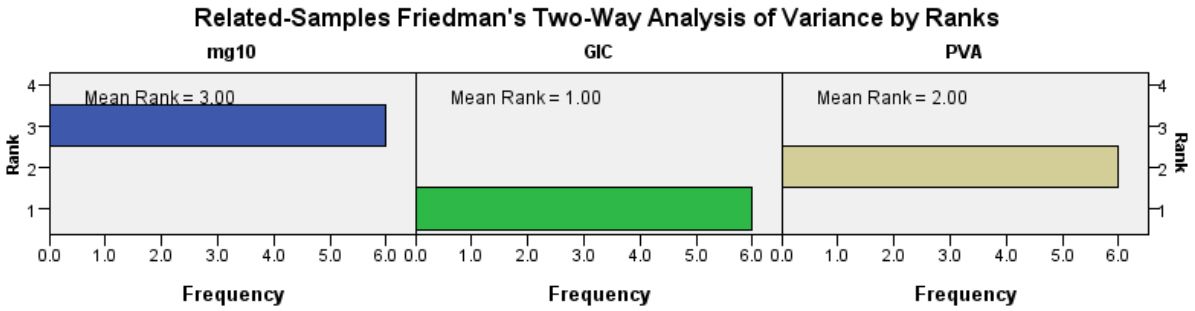
Asymptotic significances are displayed. The significance level is .05.

S. mutans CFUs related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance

10.2.7.3 Intimately attached assay anaerobic 24Hrs.

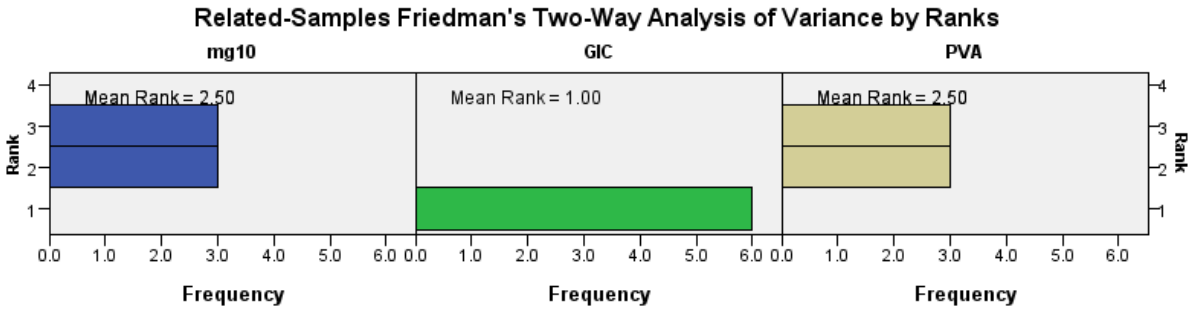
Total N	6
Test Statistic	10.333
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.006

Total CFUs related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p > 0.05$). Sig. = Significance level



Total N	6
Test Statistic	12.000
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.002

N. subflava related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance level



Total N	6
Test Statistic	9.000
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.011

S. oralis related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance level

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of mg10, GIC and PVA are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.846	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

S. mutans CFUs related Friedman's two-way ANOVA by rank test results showing no significant difference between wells contains modified and non-modified ChemiFil Superior GIC disks. ($p>0.05$). Sig. = Significance

10.3 Appendix 4. Presentations and Posters

- 24th Annual CMDN Student Symposium, Dundee, UK, 6th June 2014 (Presentation).
- Research Symposium at Crieff Hydro, UK, 25th -26th February 2016 (Poster).
- Dundee Dental School First Postgraduate Symposium, 4th June 2015 (Poster).
- Dundee Dental School Second Postgraduate Symposium, 20th May 2016 (Presentation).
- BSODR annual meeting, Plymouth, UK, 6th -8th September 2017 (Presentation).

Awards and Prizes

- Best Presentation (Third Prize), CMDN Student Symposium 2014.
- Best Poster in Dental Materials, Research Symposium at Crieff Hydro 2016.
- Best Presentation (Third Prize), Dundee Dental School Second Postgraduate Symposium 2015.